

REAL-TIME RT-PCR QUANTIFICATION OF PREGNANCY-  
ASSOCIATED PLASMA PROTEIN-A GENE EXPRESSION  
IN GRANULOSA AND THECA CELLS: EFFECTS  
OF HORMONES IN VITRO

By

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I DEDICATE THIS MANUSCRIPT.

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## **CHAPTER I**

### **INTRODUCTION**

In dairy cows, reproductive efficiency is closely related to milk production. Based on a gestation length of 285 days and a calving interval of 13.5 months, and an average daily production of 23.3 Kg of milk/cow/day (NASA, 2004), a loss in reproductive efficiency that increases the calving interval by 30 days results in a decrease in milk production of estimated value at \$193 per year. Given the total number of dairy cows in the USA, estimated in 2003 to about 9.08 million head in 23 states (NASS, 2004), the loss in milk production totals to about \$ 1.75 billion per year. This substantial loss can be prevented by better understanding reproductive efficiency and managing the post-partum interval.

The intraovarian IGF system is comprised of the ligands IGF-I and -II, their IGF type 1 and 2 receptors, several ligand binding proteins (IGFBPs) and binding protein proteases. IGF-I and -II are potent inducers of ovarian follicular steroidogenesis and mitogenesis (Spicer and Echterkamp, 1995; Spicer and Chamberlain, 1998; Spicer et al., 2002), whereas insulin-like growth factor binding proteins (IGFBPs) are inhibitors of IGF action (Spicer and Echterkamp, 1995; Spicer and Chamberlain, 1999; Rivera and Fortune, 2001, 2003a; Mazerbourg et al., 2004; Spicer 2004). IGFs (Spicer and Echterkamp, 1995; Spicer and Chamberlain, 2000) and IGFBPs (Chamberlain and

Spicer, 2001; Voge et al., 2004 ab) are produced by granulosa and theca cells in vitro and are hormonally controlled.

It is well documented that levels of total IGFs in follicular fluid are relatively constant whereas the levels of IGFBPs and the IGF receptors levels fluctuate and account for the changes in bioavailable IGFs, thus modulating IGFs action. Spicer et al. (2000) showed that increasing bioavailable IGF-I in the ovarian stroma, by infusion, increased follicular fluid estradiol concentration of small but not large follicles during the first 6 days of the estrous cycle. In fact, responsiveness of the theca or granulosa cells to bioavailable IGFs is highly dependant on the IGF type 1 receptor, since both IGF-I and -II act through IGF type 1 receptor. The numbers IGF type 1 receptors increase during differentiation (Spicer et al., 2004) and are greater in large vs. small follicle granulosa cells of cattle (Spicer et al., 1994; Stewart et al., 1996). The IGF type 2 or mannose-6 phosphate receptor function is unclear but may act to either prolong IGF-II half-life or to inactivate IGF-II via internalization and thus block its biological activity (Zhou et al., 2003; Scott and Firth, 2004).

Changes in IGFBP within the follicle control the bioavailability of IGF-I or -II (Spicer and Echterkamp, 1995; Giudice, 2001; Matsui et al., 2004; Spicer, 2004). Decreases in IGFBPs in equine, porcine, bovine and human follicles are due to differences in gene expression of the IGFBPs as well as the differences in the amount of a 200 KDa serine metalloprotease, pregnancy-associated plasma protein (PAPP)-A (Conover et al., 1999; Hourvitz et al., 2002; Monget et al., 2003; Matsui et al., 2004; Mazerbourg et al., 2004). It is suspected that PAPP-A regulates IGF bioavailability in the ovary through the degradation of primarily IGFBP-4 and -5 (Bunn et al., 2004;

Mazerbourg et al., 2004; Spicer, 2004) and therefore may be an important regulator of follicular growth and selection. PAPP-A gene expression is detectable in human granulosa cells stimulated by FSH and IGF-I (Iwashita et al., 1998), induced in rat ovaries after PMSG and hCG treatments in vivo (Hourvitz et al., 2002), and correlates with aromatase and LH receptor mRNA levels in granulosa cells of cattle and pigs (Mazerbourg et al., 2001). Santiago et al. (2005) found no difference in granulosa cell PAPP-A gene expression between dominant and subordinate follicles in cattle. By cleaving IGFBP-4 and -5, PAPP-A controls the bioavailability of IGFs, and therefore is important for the intraovarian autocrine and paracrine control of follicular development.

This review will emphasize the hormonal control of intraovarian IGFBP-4 and -5 and their protease, PAPP-A, as well as summarize research conducted to evaluate localization of PAPP-A gene expression in the ovary. Also, a section of this review will be dedicated to evaluate real-time RT-PCR as a sensitive detection method of gene expression and to rationalize our choice of this method and our normalization to 18S ribosomal RNA (18S rRNA).

## CHAPTER II

### REVIEW OF LITERATURE

#### **1. Insulin-like growth factor binding proteins and their involvement in the selection of dominant follicle**

IGF-I and –II are potent inducers of ovarian cell mitogenesis in most of the farm animal species studied to date (Monniaux and Pisselet, 1992; Spicer et al., 1993) and act with gonadotropins to synergically induce steroidogenesis (Spicer and Echterkamp, 1995; Stewart et al., 1995; Spicer et al., 2002) in granulosa and thecal cells. In cattle, levels of total IGFs in follicular fluid do not change during follicular growth (de la Sota et al., 1996; Stewart et al., 1996), whereas during follicular selection insulin-like growth factor binding proteins (IGFBP) -2, -4 and -5 decrease in healthy estrogen-dominant follicles (Stewart et al., 1996; Spicer et al., 2001; Echterkamp et al., 2004; Spicer, 2004; Santiago et al., 2005) and therefore IGFBPs are thought to be the major players in the regulation of the IGF bioavailability within the follicle. These IGFBPs have a higher affinity for IGFs than the IGF receptors and inhibit IGF action in vivo and in vitro (Giudice, 2001; Mazerbourg et al., 2004; Voge et al., 2004a). Which hormones are driving this dramatic change in IGFBP levels during growth of dominant follicles has been an active area of research. Specifically, FSH does not affect IGFBP production by bovine granulosa cells in vitro (Chamberlain and Spicer, 2001; Voge et al., 2004ab), but

decreases IGFBP production by rat granulosa cells (Zhou et al., 2003). In contrast, insulin has opposing effects on IGFBP-5 protein production within the bovine ovary, increasing IGFBP-5 production by theca and decreasing IGFBP-5 by granulosa cells (Chamberlain and Spicer, 2001). Estradiol has little effect on thecal cell IGFBP-4 but decreases IGFBP-4 production by granulosa cells of small and large bovine follicles (Spicer and Chamberlain, 2002). Although leptin and cortisol antagonize insulin-induced steroidogenesis (Spicer and Francisco, 1997; Spicer, 2001), Voge et al. (2004b) showed that neither leptin nor cortisol affect IGFBP-4 and -5 mRNA in cultured granulosa and theca cells. A 7-day intraovarian IGF-I infusion in vivo did not alter IGFBP levels in follicular fluid (Spicer et al., 2000). Recently it was found that IGF-I did not affect IGFBP-5 mRNA abundance in bovine granulosa cells, whereas IGF-I increased IGFBP-5 mRNA and protein production by bovine theca cells (Voge et al., 2004 b). Therefore, changes in IGFBP protein levels in follicular fluid may be driven by cell- and hormone-specific changes in local synthesis of IGFBP. As described in the next section, post-translational modification of IGFBP may also be involved in developmental changes in follicular fluid IGFBPs.

## **2. Proteolysis of insulin-like growth factor binding proteins**

Decreases in IGFBPs in equine, porcine, bovine and human follicles are due to differences in gene expression of the IGFBPs as well as the differences in the amount of a 200 KDa serine metalloprotease, PAPP-A (Dobashi et al., 1984; Conover et al., 1999; Hourvitz et al., 2002; Monget et al., 2003; Matsui et al., 2004; Mazerbourg et al., 2004). PAPP-A was first identified in human follicular fluid in 1984 (Sinosich et al., 1984), and is produced by numerous cell types (Lin and Halbert, 1976; Dobashi et al., 1984; Qin et

al., 2000; 2002; Mazerbourg et al., 2004). Since then, PAPP-A's proteolytic activity is thought to be an important marker of ovarian follicular growth and selection (Conover et al., 2001, Erickson and Shimasaki, 2001; Mazerbourg et al., 2001; Rhoton-Vlasak et al., 2003; Rivera and Fortune, 2003a,b). Specifically, PAPP-A is suspected to regulate IGF bioavailability in the ovary through the degradation of primarily IGFBP-4 and -5 (Chandrasekher et al., 1995; Chelius et al., 2000; Hourvitz et al., 2002; Rivera and Fortune, 2003a,b). FSH stimulates IGFBP-4 degradation in rat and human granulosa cell cultures (Shimasaki et al., 1990; Liu et al., 1993; Iwashita et al., 1998). Furthermore, IGFBP-4 proteolysis (PAPP-A) in cultured granulosa cells was induced by IGF-I or -II (Iwashita et al., 1998), which was later determined to be due to IGF binding to IGFBP-4 making IGFBP-4 more susceptible to proteolysis (Qin et al., 2000) indicating that PAPP-A proteolytic activity is dependant on IGF-I and -II which acts at a post-translational level. More recently, PAPP-A protease activity was found to be responsible for inducing ovarian surface epithelial (OSE) cell proliferation after ovulation and subsequent repair of ovulation-associated wounds (Kalli et al., 2004). Also, reduced PAPP-A mRNA expression in cancerous and immortalized ovarian surface epithelium (OSE; Kalli et al., 2004) links PAPP-A to abnormal ovarian growth. Thus PAPP-A appears to be involved with both normal and metastatic ovarian growth.

### **3. Hormonal control of pregnancy-associated plasma proteins**

#### **Hormonal control of pregnancy-associated plasma protein-A**

Using in situ hybridization, PAPP-A mRNA was detected strictly in granulosa cells of healthy small antral 5 mm to preovulatory follicles and healthy corpora lutea (Hourvitz

et al., 2000). Hourvitz et al. (2000) also reported low to non-existent PAPP-A expression in pre-antral follicles, small (1-2 mm) healthy or atretic antral follicles, large atretic antral follicles, surface epithelium, tunica albuginea and connective tissue cells. Ovarian PAPP-A mRNA was increased 4 to 36 h after mice were treated with PMSG (Hourvitz et al., 2002), and detected in rat membrana but not cumulus granulosa cells of dominant follicles in PMSG-treated rats (Matsui et al., 2004). In women, PAPP-A mRNA was detected in primary follicles, theca externa and granulosa cells of large human follicles (Rhoton-Vlasak et al., 2003). Rat ovarian PAPP-A gene expression and time course analysis using semi-quantitative RT-PCR showed PAPP-A mRNA levels increase within 12 h, peaking at 36 h to 48 h of culture while the PAPP-A protein increased only at 48 h (Matsui et al., 2004) indicating a probable delay between PAPP-A mRNA induction and PAPP-A protein production. Human PAPP-A was identified to be progesterone dependant (Bischof and Tseng, 1986) in the endometrium whereas FSH dose-dependently induced membrana granulosa cell PAPP-A gene expression in late pre-antral rat follicles (Matsui et al., 2004). Similarly PMSG increased PAPP-A gene expression in whole ovarian extracts in mice using semi-quantitative RT-PCR and northern blot analysis (Hourvitz et al., 2002). More recently, no differences in granulosa cell PAPP-A gene expression were observed between estrogen dominant and subordinate follicle granulosa cells, collected 24 or 48 h after PGF2 $\alpha$  injections (i.e. during the follicular phase) in cattle (Santiago et al., 2005), a time when high IGFBP-4 proteolytic activity exists within dominant but not subordinate follicles (Spicer et al., 2001). However, PAPP-A levels in estrogen dominant human follicles were associated with increased IGFBP-4 proteolysis and were greater than in androgen dominant follicles (Conover et al., 1999). Reasons for discrepancies among studies are unknown and will require further

elucidation.

Limited associations between PAPP-A gene expression and some of the reproductive hormones have been established. PAPP-A mRNA levels were positively correlated to aromatase and LH receptor mRNA levels in both cattle and pig granulosa cells implying estradiol and (or) LH regulation of PAPP-A (Mazerbourg et al., 2001). In contrast, granulosa cell PAPP-A mRNA and estradiol levels in follicular fluid not significantly correlated in preovulatory dominant and subordinate follicles (Santiago et al., 2005). However, serum estradiol, progesterone, and leptin levels in women were not affected by dose of GnRH agonist but intrafollicular levels of PAPP-A protein from dominant (> 20 mm) follicles were higher in women that received 400 µg vs. 200 µg of daily GnRH agonist (Suh et al., 2004). In human granulosa cells, hCG, IGF-II and a GnRH antagonist did not affect PAPP-A secretion (Conover et al., 2001; Weiss et al., 2003). Also, Ortiz et al. (2003) showed no effect of IGF-II on cultured human osteoblast expression of PAPP-A mRNA. Neither cortisol, nor estradiol affected PAPP-A secretion by human decidual explants or endometrial cells (Bischof et al., 1986; Bischof and Tseng, 1986), but progesterone stimulated PAPP-A protein production by human endometrial cells (Bischof and Tseng, 1986). cAMP induced PAPP-A mRNA in choriocarcinoma cells (Haaning et al., 1999). Clearly, more research is needed to ascertain the hormonal regulation of PAPP-A gene expression and protein secretion in ovarian and nonovarian tissues.

### **Pregnancy-associated plasma protein-A-like proteins**

Novel IGF-I-independent metalloproteinases similar to PAPP-A were identified in



mice (PAPP-Ai; Soe et al., 2002) and human (PAPP-E; Farr et al., 2000; or PAPP-A2; Overgaard et al., 2001). PAPP-A2 cleaves IGFBP-5 specifically (Overgaard et al., 2001) whereas PAPP-Ai cleaves both IGFBP-4 and -5 (Soe et al., 2002). Both PAPP-A2 (Overgaard et al., 2001) and PAPP-Ai (Soe et al., 2002) proteolytic activity is IGF independent, in contrast to the IGF-dependant PAPP-A activity (Fowles and Freemark, 1992; Conover et al., 1993; Conover et al., 1999). Furthermore, Soe et al. (2002) showed that PAPP-Ai cleavage of IGFBP-4 was considerably less than that observed by PAPP-A. Although PAPP-A and PAPP-A2/E prepro-protein show little homology in mammals, the mature protein share 45% - 62 % common residues (Farr et al., 2000; Overgaard et al., 2001). Interestingly, PAPP-A2 protein of 220 KDa does not form a dimer or bind to proform of eosinophil major basic protein (proMBP) as is the case with PAPP-A, and thus PAPP-A2 does not bind to cell surfaces like PAPP-A does (Overgaard et al., 2001; Weyer et al., 2004). Expression of PAPP-A and PAPP-Ai comparisons revealed that PAPP-A and PAPP-Ai mRNA are expressed in murine ovarian, placental and whole fetus tissues (Soe et al., 2002). Whether PAPP-A2/E and PAPP-Ai are in fact responsible for ovarian degradation of IGFBPs or whether their level of activity is under hormonal control has yet to be investigated.

#### **4. Regulators involved in pregnancy-associated plasma protein-A secretion and gene expression.**

Oocyte-derived growth factors such as growth differentiation factor (GDF)-9 and bone morphogenic protein (BMP)-15 that control FSH responses in follicular cells (Eppig et al., 1997, 2001; Erickson and Shimasaki, 2000; Matzuk, 2000) have recently been reported to regulate PAPP-A expression and secretion (Lyons et al., 1989; McGrath et al.,

1995; Elvin et al., 2000; Vitt et al., 2000; Monget et al., 2002; Matsui et al., 2004). Specifically, the ability of FSH to induce PAPP-A gene expression is inhibited by bone morphogenic protein (BMP)-15 in granulosa cells (Matsui et al., 2004). Recombinant BMP-15 inhibited the ability of FSH to stimulate PAPP-A mRNA and protein production by cultured granulosa cells, and at highest BMP-15 concentrations, PAPP-A production was almost completely suppressed (Matsui et al., 2004). Also, the eosinophil major basic protein precursor protein (proMBP) covalently and reversibly binds PAPP-A (Overgaard et al., 2000; Sivanandam et al., 2004b) and leads to the inactivation of PAPP-A proteolytic activity (Chen et al., 2002). In vitro, tumor promoters such as  $\beta$ -phorbol 12, 13-didecanoate ( $\beta$ -PDD) and Simian virus 40 induce proMBP mRNA and decrease PAPP-A mRNA after 24 h without affecting PAPP-A protein secretion (Chen et al., 2002). Moreover, transforming growth factor- $\beta$  (TGF- $\beta$ ) increases PAPP-A protein and gene expression in cultured human osteoblasts, while suppressing the proMBP expression, thus increasing bioavailable IGF-I and promoting bone proliferation (Ortiz et al., 2003). How such as proMBP interact with PAPP-A to control follicular selection remains to be elucidated.

In malignant ovarian epithelial cells (OSE), proMBP is induced, however there was no correlation between PAPP-A protein and decreased IGFBP-4 proteolysis in conditioned media from malignant ovarian cells (Kalli et al., 2004). In these ovarian tumors, increased IGF-II mRNA production was accompanied with the lowest observed PAPP-A mRNA and increased proMBP mRNA (Kalli et al., 2004). Furthermore, proMBP cDNA-transfected fibroblasts had lower IGFBP-4 proteolytic activity but unchanged PAPP-A protein synthesis (Chen et al., 2002), suggesting proMBP as a PAPP-

A activity inhibitor, rather than a PAPP-A synthesis regulator. On the other hand, PAPP-A mRNA and protein is produced by normal unmodified OSE cells, whereas proMBP levels are undetectable (Kalli et al., 2004).

In ovarian cells, proMBP was not detected in granulosa cells (Conover et al., 2001). However, Rhoton-Vlasak et al. (2003) detected higher MBP staining in theca vs. granulosa cells of large follicles, and weaker MBP staining in corpora lutea and follicular fluid from small follicles. The co-localization of PAPP-A and proMBP expression is only observed in luteal cells (Rhoton-Vlasak et al., 2003). proMBP production was not stimulated by either FSH or IGF-II in granulosa or muscle cells (Conover et al., 2001; Ortiz et al., 2003). Further elucidation is required to determine the possible role of proMBP and MBP in folliculogenesis and luteogenesis, especially through its interaction with PAPP-A.

As reviewed in this section, PAPP-A responsible for IGFBP-4 proteolysis, and is linked to the selection of the dominant ovarian follicle. Although, research on the regulators of PAPP-A gene expression or PAPP-A proteolytic activity is not extensive, the results published to date suggest that PAPP-A activity is post-transcriptionally regulated by IGFs (stimulatory) and proMBP (inhibitory) whereas PAPP-A gene expression is upregulated by FSH. Characterizing the hormonal regulation of PAPP-A gene expression in bovine ovarian tissue is the object of this thesis research. Our aim is to identify hormonal regulators of the PAPP-A gene expression in cultured granulosa and theca cells of cattle.

## **5. Real-time reverse transcription - polymerase chain reaction**

Reverse transcription (RT) followed by polymerase chain reaction (PCR) is a powerful tool for the detection and quantification of mRNA (Chelly and Kahn, 1994; Williams and Tucker, 1999; Pfaffl et al., 1998, 2002 ac), with high sensitivity (Chelly and Kahn, 1994; Bustin, 2000; Pfaffl, 2001), good reproducibility and wide quantification range (Bustin, 2000; Pfaffl, 2001). The application of fluorescence techniques to real-time PCR combines the PCR amplification product detection and qualification of newly synthesized DNA (Livak et al., 1995; Heid et al., 1996; Pfaffl et al., 1998). Furthermore, Bustin (2000) described the RT-PCR as being the most sensitive method for 1) detection and quantification of gene expression levels, in particular for low abundance mRNA from limited tissue samples and 2) elucidation of small changes in mRNA expression levels (Pfaffl et al., 1998; Bustin, 2000; Pfaffl and Hageleit, 2001). However, this sensitivity should be addressed cautiously since sometimes, the gene detection using PCR may be considered to be below physiological significance (Chelly and Kahn, 1994).

### **RT-PCR as a tool for biological detection of gene expression**

The TaqMan chemistry allows the detection of gene expression and gene differences from RNA samples. However to assure the specificity of real-time RT-PCR, primers and probes should be designed appropriately, to avoid amplification of non-specific genes. Rules for primers and probe design vary slightly in the literature (Chelly and Kahn, 1994; Williams and Tucker, 1999) or according to manufacturers such as Applied Biosystems, Qiagen, and Ambion, to name a few, recommended amplicon size

range between 70 and 150 bp, primers and probes should be screened for secondary structures and primer-dimer formation should be avoided. Runs of multiple guanine and cytosine should be avoided especially if they are on the 3' end of the both primers in order to avoid mismatched during amplification steps (Williams and Tucker, 1999). Melting temperatures of primers should be around 48°C, and the differences between the primer pairs not exceeding 2°C. The probe melting temperature should be 10°C higher than that of the primers. After the reverse-transcription step, successful quantification requires the annealing of those three oligonucleotides to the cDNA. The two template-specific primers define the endpoints of the amplicon and provide the first level of specificity. The additional specificity is provided by the use of a third oligonucleotide probe that hybridizes to the amplicon during the annealing/extension phase of the PCR (Bustin, 2000).

The TaqMan assay utilizes the 5'-nuclease activity of the DNA polymerase (AmpliTaq Gold enzymes) to cleave a oligonucleotide hybridization (TaqMan) probe bound to its target amplicon (Livak et al., 1995; Williams and Tucker, 1999; Bustin, 2000). The TaqMan probe consists of an oligonucleotide with a 5'-reporter dye and downstream, a 3'-quencher dye. The fluorescent or reporter dye, such as FAM (6-carboxy-fluorescein) or TET (tetrachloro-6-carboxy-fluorescein) is covalently linked to the 5' end of the oligonucleotide. Each of the reporters is quenched by the quencher dye, TAMRA (6-carboxy-tetramethyl-rhodamine), typically located at the 3' end. When the dual-labeled probe is intact, the proximity of the quencher and reporter dye results in suppression of the reporter fluorescence by energy transfer (Livak et al., 1995; Williams and Tucker, 1999). During PCR, forward and reverse primers hybridize to the 3' and 5'

ends of the specific target cDNA, respectively, while the TaqMan probe hybridizes specifically around the middle of the target amplicon sequence (Livak et al., 1995; Williams and Tucker, 1999; Bustin, 2000). The reporter dye and quencher dye are separated upon cleavage by the TaqMan enzyme, resulting in increased fluorescence of the reporter, when excited. The 3' end of the TaqMan probe is blocked to prevent extension of the probe during PCR (ABI, 1997; Williams and Tucker, 1999). This process occurs in every cycle and does not interfere with the exponential accumulation of the product (ABI, 1997).

The increase in fluorescence of the reporter is measured and is a direct consequence of target amplification during PCR (Livak et al., 1995; Bustin, 2000). Constant for each individual sample during the early exponential phase (Liu and Saint, 2002), the amplification efficiency is compromised during subsequent cycles (Dubois et al., 1999; Liu and Saint, 2002), mainly due to the multitude of processes involved in real-time PCR. In fact, Liu and Saint (2002) reported amount of primers, ions, nucleotides as well as enzyme activity as some of these components. The average efficiency of PCR was reported to be about 85 % during the exponential phase (Dubois et al., 1999), thus the necessity and importance of use of internal controls (Livak et al., 1995; Dubois et al., 1999; Williams and Tucker, 1999; Bustin, 2000; Liu and Saint, 2002; Janssens et al., 2004; de Kok et al., 2005) in order to correct for well to well variation due to master mix preparation and sample loading errors. The PCR reaction is however limited by the amount of reagents available for each reaction and its eventual saturation, the amplification efficiency then reaches zero (Liu and Saint, 2002).

## **Normalization and Standardization of real-time RT-PCR**

The purpose, importance and choice of internal standards were emphasized throughout the literature. Livak and Schmittgen (2001) identified the selecting and validating an appropriate internal control gene as the first step in the design and evaluation of an experiment using RT-PCR. The purpose of use of internal standards is mainly to provide a known copy of template in quantitative PCR, and to serve as a positive control for both RNA extraction and the amplification step in quantitative assays (Pfaffl et al., 1998; Dubois et al., 1999, de Kok et al., 2005). Normalization to an endogenous reference is necessary to correct results for differing amounts of input RNA and to determine the effect of the experimental treatment of a candidate internal control gene (Pfaffl et al., 1998; Livak and Schmittgen, 2001; Pfaffl et al., 2005).

The use of internal standards solves problems of variation in starting amounts of template and operator loading errors (Crawford et al., 2001; Bustin, 2002) but proper validation of internal control genes is necessary when designing quantitative gene expression studies (Schmittgen and Zakrajsek, 2000; Livak and Schmittgen, 2001; Janssens et al., 2004; de Kok et al., 2005) and for each experiment to determine that gene expression is unaffected by the experimental treatment (Pfaffl et al., 1998; Livak and Schmittgen, 2001; Schmittgen et al., 2001). Others recently suggested the use of multiple housekeeping genes to normalize gene expression data especially from microarray data and suggested multitude of mathematical models to accommodate such corrections (for review: Tricarico et al., 2002; Vandesompele et al., 2002; Pfaffl et al., 2002c; 2004; Janssens et al., 2005). Standard housekeeping genes usually suffice as internal control genes (Pfaffl et al., 1998; Dubois et al., 1999; Livak and Schmittgen, 2001; Liu and Saint,

2002; Goidin et al., 2002).

Other methods for normalization have been evaluated; Bustin (2000) showed that the normalization to total cellular RNA is the most reliable method. However, little is known about the total RNA content per cell of different tissues in vivo, or how it might vary between normal and tumor tissues (Bustin, 2002). Normalization to total RNA content requires accurate quantification of the RNA sample by either the RiboGreen ([www.probes.com](http://www.probes.com)), RNA quantification assay that relies on a dye that exhibits significant fluorescence enhancement on binding to nucleic acids, or the OD<sub>260</sub> reading from a spectrophotometer. However in small samples, it is important to normalize to internal standards due to the difficulty of quantification of total RNA from these samples. In vivo, mRNA levels of all housekeeping genes likely vary to such degree that normalization becomes inaccurate and or misleading (Bustin, 2002; Bustin and Dorudi, 2002; de Kok et al., 2004; Bas et al., 2004; Janssens et al., 2005).

Knowing that rRNA makes the bulk of a total RNA sample, rRNA was proposed as an alternative normalizer, despite reservations concerning its expression levels, transcription by a different RNA polymerase and possible imbalances in rRNA and mRNA fractions between different samples (Bustin, 2002), levels of rRNA remain constant during serum-stimulation of cultured cells (Schmittgen and Zakrajsek, 2000; Goidin et al., 2001) and its use has been validated on several tissues (Bustin, 2002; Schmittgen and Zakrajsek, 2000; Schmittgen et al., 2001;) including muscle (Jemiolo and Trappe, 2004), melanoma (Goidin et al., 2001; de Kok et al., 2005), kidney tissue (Schmid et al., 2003), and T-lymphocytes (Bas et al., 2004) as well as disease states such as diabetic glomerulosclerosis (Biederman et al., 2004) and cancer research (Janssens et



al., 2004).

Housekeeping genes are so called because their synthesis, necessary for the cell survival, occurs in all nucleated cell types, and thus synthesis of these genes is often considered not to fluctuate as much as the target genes (Thellin et al., 1999; Livak and Schmittgen, 2001; Janssens et al., 2004; de Kok et al., 2005). Ideally, the internal control gene for quantitative gene expression studies should not be influenced by the conditions of the experiment (Livak and Schmittgen, 2001; Janssens et al., 2004; de Kok et al., 2005). The most often considered and used housekeeping genes are  $\alpha$ - and  $\beta$ -actin, cyclophilin,  $\alpha$ - and  $\beta$ -tubulins, hypoxanthine phosphoribosyl transferase (HRPT), L32 (Thellin et al., 1999) or 18S rRNA (Dubois et al., 1999; Thellin et al., 1999; Goidin et al., 2001), 28S rRNA and  $\beta_2$ -microglobulin (Thellin et al., 1999; Livak and Schmittgen, 2001). Schmittgen and Zakrajsek (2000) demonstrated that the serum-stimulation influenced the expression of several commonly used housekeeping genes. In fact, serum-stimulation for 8 h significantly increased the expression of  $\beta$ -actin and GAPDH (Schmittgen and Zakrajsek, 2000). Furthermore, serum stimulated the expression of  $\beta$ -actin and GAPDH to a greater extent than it influenced cellular mRNA levels (Schmittgen and Zakrajsek, 2000). Also, Goidin et al. (2001) found that GAPDH mRNA levels are highly heterogeneous even in cellular sub-populations of the same pathological order. Bustin (2002) found that the GAPDH mRNA levels are not constant and GAPDH contributes to diverse cellular functions such as nuclear RNA export, DNA replication, and DNA repair. In addition, Selvey et al. (2001) reported that  $\beta$ -actin showed to be highly regulated by matrigel. However, no relationship existed between the time of serum stimulation and expression of 18S rRNA (Schmittgen and Zakrajsek, 2000). More

recently, Bas et al. (2004) found that levels of  $\beta$ -actin and GAPDH fluctuated with status of human peripheral blood mononuclear cells (PBMC) as opposite to 18S rRNA, and showed 30-70 fold increase of GAPDH upon activation of PBMCs. Therefore, for most experimental conditions,  $\beta$ -actin and GAPDH should not be used as internal controls and normalization to these internal controls is inappropriate (Schmittgen and Zakrajsek, 2000; Goidin et al., 2001; Selvey et al., 2001; Bustin, 2002), whereas 18 S rRNA may be used safely as an internal control in quantitative gene expression studies of serum-stimulated fibroblasts only when the reverse transcription contain equal amounts of RNA (Bustin, 2002). Selvey et al. (2001) reported that the 18S rRNA demonstrated excellent consistency, reproducibility and non-regulation by a matrigel treatment competitor technology, includes blocked internal standard primers such as 18 S rRNA or  $\beta$ -actin to act as competitors in the PCR reaction. The sole purpose of these competitors is to attenuate the internal control amplification, and therefore provides a more accurate validation. Goidin et al. (2001) stated that the relative RT-PCR procedure including ribosomal 18 S rRNA as internal standard and competitor technology is precise for RNA quantification and is tailored for cDNA array validation. Therefore, since RT-PCR requires the use of minimally regulated housekeeping genes (Dubois et al., 1999; Selvey et al., 2001; Liu and Saint, 2002),  $\beta$ -2 microglobulin and 18S rRNA are suitable internal control genes in quantitative serum-stimulation studies, while GAPDH and  $\beta$ -actin are not (Schmittgen and Zakrajsek, 2000).

### **Calculation Methods for real-time RT-PCR**

In RT-PCR, fluorescence values recorded during every cycle, represent the amount of product amplified to the point in which the fluorescence signal is first recorded

as statistically significant above the background (Gibson et al., 1996). This point is defined as the threshold cycle ( $C_t$ ) and will always occur during the exponential phase of amplification (Bustin, 2000).  $C_t$  is therefore determined from a log-linear plot of the PCR signal versus the cycle number (Gibson et al., 1996; ABI, 1997; Bustin, 2000). Because  $C_t$  is an exponential and not a linear term, any statistical presentation using the raw  $C_t$  should be avoided (Gibson et al., 1996; Bustin, 2000; Schmittgen and Zakrajsek, 2000; Pfaffl and Hageleit, 2001). Absolute quantification method expresses the absolute mRNA copy number per vial or capillary that is determined by comparison with appropriate external calibration curve (Pfaffl and Hageleit, 2001; Pfaffl et al., 2002 a). An absolute quantification makes it easier to compare expression data among different runs and laboratories, because the calibration curve is non-changing, and reliable (Pfaffl et al., 2002 a). The relative expression is based on the expression ratio of a target gene versus a reference gene and is adequate for most purposes to investigate physiological changes in gene expression levels (Pfaffl and Hageleit, 2001; Pfaffl et al., 2002 a).

Presentation of the relative PCR data is often calculated along with an internal control and (or) calibration sample, and is rarely presented in examining the sample-to-sample variation among replicate reactions. Converting the individual data to a linear form using  $2^{-C_t}$  more accurately depicts the individual variation among replicate reactions (Livak and Schmittgen, 2001). Trends can be better explained by relative quantification, but the results are strongly dependant on the reference gene and normalization procedure used (Pfaffl et al., 2002 ac). The choice of calibration for the  $2^{-\Delta\Delta C_t}$  method depends on the type of planned gene expression experiment (Livak and Schmittgen, 2001). Equations and models used for the calculation of relative expression

ratios allow for the determination of only a single transcription difference between one control and one sample (Pfaffl et al., 2002 ac). The simplest design is to use the untreated control as the calibrator, and the data is then expressed as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control where  $\Delta\Delta C_t=0$  and  $2^0=1$  (Livak and Schmittgen, 2001). More recently, researchers have further modified the  $2^{-\Delta\Delta C_t}$  data by expressing the experimental data set relative to the lowest fold gene expression values (Hettinger et al., 2001; Ross et al., 2003; Voge et al., 2004ab).

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## Chapter III

# REAL-TIME RT-PCR QUANTIFICATION OF PREGNANCY-ASSOCIATED PLASMA PROTEIN-A GENE EXPRESSION IN GRANULOSA AND THECA CELLS: EFFECTS OF HORMONES IN VITRO

### 1. Abstract

Ovarian follicular growth and dominance is controlled by a series of hormonal events including a decrease in intrafollicular IGF-binding proteins (IGFBP)-2, -4 and -5 levels. Proteolytic enzymes such as pregnancy-associated plasma protein-A (PAPP-A) degrade IGFBPs and increase bioavailability of IGF-I and -II during follicular development. The objective of this study was to determine the effect of IGF-I, IGF-II, insulin (INS), LH, FSH, estradiol (E), leptin or cortisol on ovarian PAPP-A mRNA levels. Granulosa (GC) from small (SM) (<5 mm) and large (LG) (>7.9 mm) follicles as well as theca cells (TC) from LG follicles were collected from bovine ovaries and cultured for 48 h in medium containing 10% FCS and then treated with various hormones in serum-free medium for an additional 12 or 24 h. For time course analysis, LG-GC and LG-TC were cultured for either 12 or 24 h with 0 or 100 ng/mL of IGF-I or -II (exp 1 and 2). In all other experiments, SM-GC, LG-GC and LG-TC were treated with various

combinations of IGF-I, IGF-II, FSH, LH, E, INS, leptin and (or) cortisol for 24 h (exp 3 through 12). PAPP-A mRNA expression levels quantified using real-time RT-PCR. In SM-GC and LG-GC, none of the treatments significantly affected ( $P > 0.10$ ) PAPP-A gene expression. In LG-TC, IGF-I, IGF-II (averaged across exp 2, 5 and 7), LH or cortisol did not affect ( $P > 0.10$ ) PAPP-A mRNA levels, whereas INS with or without LH decreased ( $P < 0.05$ ) PAPP-A mRNA by 2-fold; E alone and in the presence of insulin decreased thecal PAPP-A mRNA levels and amplified the insulin-induced inhibition of PAPP-A mRNA expression. We conclude that PAPP-A gene expression is differentially regulated in granulosa and theca cells. Insulin alone and in combination with estradiol decreased thecal PAPP-A mRNA levels which would likely reduce the bioavailable IGFs in the theca layer during growth and selection of follicles. Thus, estradiol may act as a negative feedback regulator to prevent excessive IGF-I – induced androgen production, and hence prevent excessive estradiol production by granulosa cells via decreased thecal PAPP-A production, maintaining desirable levels of IGFBP-4 and -5 and subsequently limiting free IGF-I /-II within the follicle during follicular development.

**Keywords:** Insulin-like growth factor binding protein (IGFBP); granulosa cell; theca cell; pregnancy-associated plasma protein-A (PAPP-A).

## **2. Introduction**

Ovarian follicular growth and dominance are controlled by a series of hormonal events that lead to changes in granulosa and thecal cell gene expression of various intrafollicular regulatory proteins including the insulin-like growth factor binding proteins (IGFBPs) (Rivera and Fortune, 2001; Spicer and Chamberlain, 2002;

Mazerbourg et al., 2003; Mazerbourg et al., 2004; Spicer, 2004). Because IGF-I and -II are potent inducers of ovarian follicular steroidogenesis and mitogenesis (Spicer and Echtenkamp, 1995; Spicer and Chamberlain, 1998; Spicer et al., 2002) and IGFBPs are inhibitors of IGF action (Spicer and Chamberlain, 1999; Voge et al., 2004a), changes in IGFBP within the follicle control the bioavailability of IGF-I or -II and thus control follicular development (Spicer, 2004). During follicular selection, IGFBP-2, -4 and -5 decrease in healthy estrogen-dominant follicles (Stewart et al., 1996; Chandrasekher et al., 1995; Conover et al., 1999; Spicer et al., 2001; Echtenkamp et al., 2004; Spicer, 2004; Santiago et al., 2005). Recent reports indicate that the decreases in IGFBPs in equine, porcine, bovine and human follicles are due to differences in gene expression of the IGFBPs as well as the differences in the amount of a 200 KDa serine metalloprotease, PAPP-A (Conover et al., 1999; Laursen et al., 2001; Lawrence et al., 1999; Monget et al., 2003; Mazerbourg et al., 2004), and thus PAPP-A may be an important regulator of follicular growth and selection. PAPP-A gene expression is detectable in human granulosa cells stimulated by FSH and IGF-I (Iwashita et al., 1998), induced in rat ovaries after PMSG and hCG treatments in vivo (Hourvitz et al., 2002), and correlates with aromatase and LH receptor mRNA levels in cattle and pigs (Mazerbourg et al., 2001). In contrast, Santiago et al. (2005) found no difference in granulosa cell PAPP-A gene expression between dominant and subordinate follicles in preovulatory cattle. However, little or no research has been conducted on the hormonal regulation of PAPP-A gene expression in cultured follicular cells. Investigation of the hormonal control of PAPP-A mRNA in granulosa and theca cells may reveal possible regulatory mechanisms involved in the control of the PAPP-A/IGF system. Our objective was to determine the effect of INS, IGFs, E, LH, FSH, leptin and (or) cortisol on PAPP-A gene expression in

cultured bovine granulosa and theca cells.

### **3. Materials and Methods**

#### **Reagents and hormones**

The reagents used in cell culture were: Dulbecco modified Eagle medium (DMEM), Ham's F-12, sodium bicarbonate, gentamicin, trypan blue, fetal calf serum (FCS), protease, collagenase, hyaluronidase, and DNase from Sigma Chemical Company (St. Louis, MO).

The reagents used in RNA extraction were: Trizol reagent from Life Technologies, Inc. (Gaithersburg, MD), chloroform from Sigma Chemical Co., (St. Louis, MO), isopropyl alcohol (Pierce Chemical Company (Rockford, IL), RiboGreen<sup>®</sup> RNA Quantitation Reagent and Kit from Molecular Probes (Eugene, OR)

The hormones used in cell culture were: LH (bovine L1914, LH activity 2.0 x NIH-LH-S1 U/mg) and FSH (ovine F1913, FSH activity, 15 x NIH-FSH-S1 U/mg) from Scripps Laboratories (San Diego, CA); recombinant mouse leptin from Repro Tech Inc. (Rocky Hills, NJ); recombinant human insulin-like growth factor (IGF) -I and -II from R&D Systems (Minneapolis, MN); and FCS, purified bovine insulin (28 U/mg) and estradiol from Sigma Chemical Co. (St. Louis, MO).

#### **Cell Culture**

Ovaries from pregnant and non-pregnant dairy and beef cows were collected from a local abattoir and were transported to the lab in 0.9% saline solution at 4 C. Follicular

fluid from small (1-5 mm) and large-follicles (>7.9 mm) was aspirated using a 20 gauge needle and a 3 mL syringe and centrifuged at 200 x g for 5 min to isolate granulosa cells as previously described (Langhout et al., 1991; Spicer and Chamberlain, 1998; Spicer et al., 2002). Granulosa cells were re-suspended in serum-free media containing collagenase and DNase (Sigma Chemicals Company, St. Louis, MO) at 1.25 mg/mL and 0.5 mg/mL, respectively, to prevent cell clumping.

Theca cells were collected from large-follicles as previously described (Stewart et al., 1995; Spicer and Chamberlain, 1998). Briefly, large-follicles were bisected longitudinally after aspiration of follicular fluid and granulosa cells separated from the theca interna via blunt dissection, and combined with the original corresponding pool of large-follicle granulosa cells isolated from follicular fluid. The theca interna tissue was torn apart using two rat-tooth forceps and enzymatically digested for 1 h at 37 C on a rocking platform. The non-digested tissue was removed via filtration through sterile syringe filter holder with a metal screen of 149  $\mu$ m mesh (Gelman Sciences, Ann Arbor, MI). Theca cells were then centrifuged at 50 x g for 5 min, supernatant discarded, and pellet washed with serum-free media. The theca cells were re-suspended in the same media as described for the granulosa cells.

Viability of granulosa cells from small and large follicles and of theca cells from large follicles was determined by trypan blue exclusion method on a 0.1mm deep hemacytometer (American Optical Corporation, Buffalo, NY), and averaged:  $42.6 \pm 15.7$ ,  $73.0 \pm 11.7$ , and  $87.6 \pm 5.6\%$ , respectively.

Approximately  $2.0 \times 10^5$  viable cells were plated on 24-well Falcon multiwell

plates (No. 3047; Becton Dickinson, Lincoln Park, NJ) in a 1mL of mixture of 1:1 Dulbecco modified Eagle medium and Ham's F-12 containing 0.12 mM and 2.0 mM of gentamycin and glutamine, respectively, as well as 38.5 mM sodium bicarbonate. Cells were cultured in an environment of 95% air and 5% CO<sub>2</sub> at 38.5 C in 10 % fetal calf serum (FCS) for the first 48 h with a medium change at 24 h. Cells were then washed twice with serum-free media and the different hormonal treatments applied in serum-free media for 12 h or 24 h depending on the experiment.

### **RNA extraction**

At the end of the culture, medium was aspirated from each well and cells from 2 replicate wells were lysed in 0.5 mL of Trizol<sup>®</sup> Reagent (Life Technologies Inc., Gaithersburg, MD). Briefly, 0.5 mL of Trizol was added to the first well, cells lysed by repeated pipetting and transferred to the replicate well, and then into a 1.5 mL eppendorf tube. Each treatment containing 4 wells generated 2 replicated samples of RNA. Cell lyses was incubated in Trizol for 5 min at 22 C, then 0.10 mL chloroform added to each sample that was vortexed for 15 sec. After a 2 to 3 min incubation at room temperature, samples were centrifuged at 3500 x g, 4 C for 30 min using eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY). The upper aqueous phase was then transferred to a new eppendorf tube and RNA precipitated with 0.250 mL of isopropanol and samples gently mixed by inversion. RNA was pelleted after a 10 min incubation followed by a centrifugation at 3500 x g, 4 C for 10 min. The RNA pellet was washed with 0.5 mL of 70 % ethanol and allowed to dry for 6 min at room temperature. The RNA pellet was dissolved in 0.3 mL of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.4).

RNA was quantitated by spectrophotometry at 260 nm or via an ultra-sensitive fluorescent nucleic acid staining using RiboGreen® (Molecular Probes Inc., Eugene, OR) following manufacturer's specifications with modifications. Aliquot of RNA and the RNA standard (1000 g/mL) were diluted 50 fold with 1x TE buffer (supplied in the Kit) and the RiboGreen® RNA quantitation reagent was diluted 200-fold. A standard dilution curve was then generated from the 50-fold diluted RNA standard to the following final concentrations: 1000, 750, 500, 250, 100, 75, 25, 10, and 0 ng/mL. The RNA sample and standard was pipetted in black 100 L 96-well microplates (Proxiplate™-96F, P/N 6006270, Packard Bioscience BV, Meridian, CT), followed by the aqueous working solution of RiboGreen® RNA quantitation reagent. After 5 min dark incubation at 25 °C, RiboGreen-bound RNA fluorescence was determined via top reading using a Wallac 1420 (Perkin-Elmer, Boston, MA) at 500 nm maximum excitation and 525 nm maximum emission. The fluorescence value of the reagent blank (0 ng/mL RNA) was subtracted from each of the samples. Fluorescence of the standard RNA was plotted with its corresponding concentrations. Given the adjusted fluorescence value of the samples, RNA concentrations were determined. The intraassay coefficient of variation was 17%.

### **Primers and Probe Design**

PAPP-A primers and probes for quantitative RT-PCR were designed using Primer Express™ software (Foster City, CA) with the following manufacturer's restrictions: primers melting temperature (T<sub>m</sub>) was set to 50 to 60 °C, whereas the probe's T<sub>m</sub> was at least 10 °C higher. The GC base pair content for the primers and probe was between 20 to 80% to avoid runs of identical nucleotides. The length of the strand was between 9 and

30 nucleotides. The PAPP-A (851 bp, Accession AF421141) forward primer was constructed between bp 557 and 578 with a T<sub>m</sub> of 59 °C and had a sequence of CAGATGTTGAGCAGCCCTGTAA. The reverse primer, TGGGTTGACGGCTGAATTGG, was constructed between bp 602 and 620 with a T<sub>m</sub> of 60 °C. The PAPP-A probe was isolated between bp 581 and 600 with a T<sub>m</sub> of 69 °C, a sequence of CCAGCGTCCGCACCTGGAGC having a FAM and TAMRA as the 5' reporter and 3' quencher dyes, respectively. All aforementioned primers met the literature reviewed (Chapter II - section 5) and manufacturer's (listed above) criterium regarding GC content and melting temperature. A "short, nearly exact matches" BLAST query search (<http://www.ncbi.nlm.nih.gov/BLAST>) was also conducted to insure the specificity of the designed primers and probe and to assure that they were not designed from any homologous regions, coding for other genes.

The differential expression of PAPP-A in granulosa or theca cells was quantified using the one-step real-time RT-PCR reaction following manufacture's specifications with modifications for Taqman® Gold RT-PCR Kit (P/N N808-02333; PE Biosystems, Foster City, CA) as previously described (Voge et al., 2004ab; Santiago et al., 2005). Briefly, After the optimization run, and based on its results, 100 ng of total RNA was amplified in a total reaction volume of 25 µL consisting of 200 nM forward primer, 200 nM reverse primer and 200 nM fluorescent probe for PAPP-A, along with 12.5 µL of TaqMan Master Mix without uracil N-glycosylase, and 1U Multiscribe with RNase inhibitor (P/N 4309169, Applied Biosystems, Foster City, CA), the volume was added up with DEPC-treated water. On the same microplate as the template, 100 pg of total RNA sample were loaded in a 25 µL total reaction volume containing 12.5 µL of TaqMan



Master Mix without UNG, 40 U Multiscribe with RNase inhibitor (P/N 43096169, Applied Biosystems, Foster City, CA) and 100 nM of the supplied 18S rRNA forward and reverse primers as well as the 18S rRNA probe labeled with VIC/TAMRA dyes.

Thermal cycling conditions were set to 30 min at 48.8°C for reverse transcription, 95°C for 10 min for AmpliTaq Gold Activations, and finished with 40 cycles at 95°C for 15 sec for denaturing and 60°C for 1 min for annealing and extension. Ribosomal 18S RNA (18S rRNA) control kit (P/N 4308329, PE Biosystems, Foster City, CA) was used as internal control to normalize samples for the variation in amounts of RNA loaded.

Furthermore, the RT-PCR product was run on a 3% Agarose I gel (AMRESCO Inc., Solon Industrial, OH) with 5 µL ethidium bromide (100 mg/mL) and a lane with PCR markers (Promega, Madison, WI) in a DNA size standard of 50 bp to 1000 bp. The observed imaging verified the length and size of the target gene. Furthermore, 6 µL of the same RT-PCR cDNA sample (from a total of 25 µL RT-PCR product after 42 cycles) was treated with 0.5 µL of shrimp alkaline phosphatase (E70092Y, Amersham Biosciences, Piscataway, NJ) and 0.5 µL exonuclease I (E700732, Amersham Biosciences, Piscataway, NJ), incubated at 37°C for 30 min and further incubated at 85°C for 15 min before sequencing, to verify the sequence and specificity of the RT-PCR quantification of the desired target gene.

Quantification of gene expression was done by setting an arbitrary threshold (Ct) on the FAM or VIC curves in the geometric portion of the RT-PCR amplification plot after examining the log view. Relative quantification of PAPP-A mRNA was expressed using the comparative threshold cycle method (Livak and Schmittgen, 2001; ABI, 1997).

Briefly, the  $\Delta C_t$  was determined by subtracting the 18S Ct value from the target unknown value. Within each experiment, the  $\Delta\Delta C_t$  was determined by subtracting the higher  $\Delta C_t$  (the least expressed unknown) from all other  $\Delta C_t$  values. Fold changes in mRNA expression were calculated as being equal to  $2^{-\Delta\Delta C_t}$ .

## **Experimental Design**

**Experiment 1** was designed to test the effect of time and IGF-I on PAPP-A mRNA in large-follicle granulosa cells. Cells were cultured for 48 h in 10% FCS then washed with serum-free media with the treatments applied for either 12 or 24 h as follows: 0 h (cells collected directly after wash with serum free media), 12 h (cells cultured for 12 h in serum-free medium), 12 h plus IGF-I (100 ng/mL), 24 h (cells were cultured for 24 h in serum-free medium) and 24 h plus IGF-I (100 ng/mL). At the end of the experiment (12 or 24 h), cells were lysed at the defined times in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 2** was designed to test the effect of time and IGF-II on PAPP-A mRNA in large-follicle theca cells. Cells were cultured as described earlier (experiment 1) with treatments applied for either 12 or 24 h as follows: 0 h (cells collected directly after wash with serum free media), 12 h (cells cultured for 12 h in serum-free medium), 12 h plus IGF-II (100 ng/mL), 24 h (cells were cultured for 24 h in serum-free medium) and 24 h plus IGF-II (100 ng/mL). At the end of the experiment (12 or 24 h), cells were lysed at the defined times in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 3** was designed to compare the effect of IGF-I and -II on PAPP-A mRNA in small-follicle granulosa cells. Cells were cultured for 48 h in 10% FCS,

washed twice with serum-free media as described earlier, and the following three treatments applied for 24 h: control (no hormones), IGF-I (100 ng/mL) or IGF-II (100 ng/mL). After 24 h, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 4** was designed to compare the effects of IGF-I, IGF-II, FSH and leptin on PAPP-A mRNA in large-follicle granulosa cells. Cells were cultured as described in experiment 3 with the following six treatments: control (no hormones), low IGF-I (3 ng/mL), high IGF-I (100 ng/mL), high IGF-II (100 ng/mL), FSH (30 ng/mL) or leptin (100 ng/mL). After 24 h, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 5** was designed to compare the effect of IGF-I and -II on PAPP-A mRNA in large-follicle theca cells. Cells were cultured as described in experiment 3 with the following three treatments: control (no hormones), IGF-I (100 ng/mL) or IGF-II (100 ng/mL). After 24 h, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 6** was designed to compare the effect of IGF-I and FSH on small-follicle granulosa cells. Cells were cultured as described in experiment 3 with the following six treatments applied for 24 h: control (no hormones), 100 ng/mL of IGF-I, 3 ng/mL of FSH, or 100 ng/mL of IGF-I plus 3 ng/mL of FSH. At the end of the culture, cells were lysed in 0.5 mL Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 7** was designed to compare the effects of IGF-II and estradiol on large-follicle theca cells. Cells were cultured as described for experiment 3 with the

following six treatments: control (no hormones), 100 ng/mL of IGF-II, 3 or 300 ng/mL of estradiol, or IGF-II plus either 3 or 300 ng/mL of estradiol. After 24 h, cells were lysed in 0.5 mL Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 8** was designed to compare the effect of estradiol, FSH and LH on PAPP-A mRNA expression in small-follicle granulosa cells. Cells were cultured as described in experiment 3 with the following six treatments: control (no additives), FSH (30 ng/mL), LH (30 ng/mL), estradiol (300 ng/mL), estradiol (300 ng/mL) plus LH (30 ng/mL), or estradiol (300 ng/mL) plus FSH (30 ng/mL). After 24 h of treatment, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 9** was designed to compare the effect of LH, FSH and estradiol in the presence of insulin. Small-follicle granulosa cells were cultured as described in experiment 3 with the following six treatments: insulin (100 ng/mL; Control), insulin plus low FSH (3 ng/mL), insulin plus high FSH (30 ng/mL), insulin plus LH (30 ng/mL), insulin plus high FSH plus low estradiol (3 ng/mL), or insulin plus high FSH plus high estradiol (300 ng/mL). After 24 h, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 10** was designed to compare the effect of LH, FSH and estradiol on PAPP-A mRNA expression in large-follicle granulosa cells in the presence of insulin. Cells were cultured as described in experiment 3 with the following six treatments: control (no hormones), insulin (100 ng/mL), insulin plus LH (30 ng/mL), insulin plus FSH (30 ng/mL), insulin plus FSH plus low estradiol (3 ng/mL), or insulin plus FSH plus high estradiol (300 ng/mL). After 24 h, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA

extraction as described earlier.

**Experiment 11** was designed to compare the effect of insulin, LH and estradiol on PAPP-A mRNA in large-follicle theca cells. Cells were cultured as described in experiment 3 with the following six treatments: control (no additives), insulin (100 ng/mL), LH (30 ng/mL), LH plus insulin, estradiol (300 ng/mL), or estradiol plus insulin. After 24 h, cells were lysed in 0.5 mL of Trizol<sup>®</sup> for RNA extraction as described earlier.

**Experiment 12** was designed to determine the effect of cortisol on large-follicle theca cell PAPP-A mRNA. Large-follicle theca cells were cultured as described in experiment 3 with the following three treatments: 0 ng/mL of cortisol plus 100 ng/mL of insulin, 3 ng/mL of cortisol plus 100 ng/mL of insulin, and 30 ng/mL of cortisol plus 100 ng/mL of insulin.

Steroid hormones (i.e. estradiol and cortisol) were diluted in 100 % Ethanol before being dissolved in the culture media. In the experiments where steroids were included as treatments, identical volumes of ethanol were added to control and steroid-treatment media in order to cancel any effect ethanol might have on cell function. Final ethanol concentrations did not exceed 0.03 % in any of the experiments.

### **Statistical Analysis**

At least 3 to 4 different pools of granulosa and theca cells were used as experimental replicates. Each pool of large-follicle granulosa and theca cells was obtained from five to seven follicles. Each pool of small-follicle granulosa cells was obtained from approximately 5 to 15 ovaries within each experimental replicate.

Treatments were applied to four different wells, and duplicate samples for each pool of cells were derived by combining RNA from two wells. The treatment effects on the dependant variables (e.g. PAPP-A mRNA level) were determined using ANOVA and the general linear models procedure of SAS for Windows (version 8.02, SAS Institute Inc., Cary-NC, USA, 1999-2001). Outliers were detected according to the procedure described by Grubbs (Grubbs, 1950). Main effects included treatment and experimental replicate and their interactions. Mean differences were determined by Fisher's protected least significant differences test, if significant treatment effects in ANOVA were detected. Data were presented as the least square means ± SEM.

#### **4. Results**

##### **Experiment 1: Effect of time and IGF-I on large-follicle granulosa cell PAPP-A mRNA.**

PAPP-A mRNA was detectable in large-follicle granulosa cells, but neither IGF-I or time of sample collection affected ( $P > 0.10$ ) PAPP-A mRNA levels (Figure 1). The Ct and  $\Delta$ Ct values are summarized in table 1.

##### **Experiment 2: Effect of time and IGF-II on large-follicle theca cell PAPP-A mRNA expression**

Large-follicle theca cells expressed detectable amounts of PAPP-A mRNA by real time RT-PCR. Thecal PAPP-A gene expression was increased ( $P < 0.05$ ) threefold between 12 and 24 h in IGF-II – treated cultures (Figure 2). However, PAPP-A mRNA levels did not change ( $P > 0.10$ ) between 0 and 24 h in the absence of IGF-II (Figure 2).

The Ct and  $\Delta$ Ct values are summarized in table 1.

**Experiment 3: Effect of IGF-I and -II on PAPP-A mRNA in small-follicle granulosa cells.**

Real-time fluorescent RT-PCR detected measurable amounts of PAPP-A mRNA in small-follicle granulosa cells, but neither 100 ng/mL of IGF-I nor 100 ng/mL of IGF-II affected ( $P > 0.10$ ) small-follicle granulosa PAPP-A mRNA levels (Table 2). The Ct and  $\Delta$ Ct values are summarized in table 2.

**Experiment 4: Effects of IGF-I, IGF-II, FSH and leptin on PAPP-A mRNA in large-follicle granulosa cells.**

Concentrations of 3 or 100 ng/mL of IGF-I, or 100 ng/mL of IGF-II, FSH and leptin did not affect ( $P > 0.10$ ) large-follicle granulosa PAPP-A mRNA levels (Table 2). The Ct and  $\Delta$ Ct values are summarized in table 2.

**Experiment 5: Effect of IGF-I and -II on PAPP-A mRNA in large-follicle theca cells.**

At 100 ng/mL, IGF-II but not IGF-I decreased ( $P < 0.05$ ) thecal PAPP-A gene expression by 39% and 21 % below control and IGF-I levels, respectively (Figure 3). The Ct and  $\Delta$ Ct values are summarized in table 2.

**Experiment 6: Effect of IGF-I and FSH on PAPP-A mRNA expression in small-follicle granulosa cells.**

Treatment with 100 ng/mL of IGF-I, 30 ng/mL of FSH or their combination did not affect ( $P > 0.10$ ) small-follicle granulosa cell PAPP-A mRNA levels. The Ct and  $\Delta$ Ct

values are summarized in table 2.

**Experiment 7: Effects of IGF-II and estradiol on PAPP-A mRNA expression in large-follicle theca cells.**

Alone, estradiol at 3 ng/mL and IGF-II at 100 ng/mL did not affect ( $P > 0.10$ ) PAPP-A mRNA levels in large-follicle theca cells, but the combination of 3 ng/mL of estradiol and IGF-II suppressed ( $P < 0.05$ ) PAPP-A gene expression by 26 % compared with control (Figure 4). Higher doses of estradiol (300 ng/mL) alone or combined with IGF-II decreased ( $P < 0.05$ ) PAPP-A gene expression by 33 and 34 %, respectively from the control levels but these levels were not significantly different from 3 ng/mL of estradiol plus IGF-II (Figure 4). The Ct and  $\Delta$ Ct values are summarized in table 2.

**Experiment 8: Effect of FSH, LH and estradiol on PAPP-A mRNA expression in small-follicle granulosa cells.**

Alone, 30 ng/mL of FSH or LH, or 300 ng/mL of estradiol did not affect ( $P > 0.10$ ) small granulosa cell PAPP-A gene expression as compared to controls (Table 3). Also, LH plus estradiol or FSH plus estradiol did not affect ( $P > 0.10$ ) PAPP-A mRNA levels. The Ct and  $\Delta$ Ct values are summarized in table 3.

**Experiment 9: Effect of LH, FSH and estradiol on PAPP-A mRNA expression in small-follicle granulosa cells in the presence of insulin.**

Small-follicle granulosa cell PAPP-A gene expression was not affected ( $P > 0.10$ ) by any of the doses of estradiol, FSH or LH tested in the presence of insulin (Table 3). Also, estradiol plus FSH or estradiol plus LH showed no suppressive or inductive ( $P >$



0.10) effect on small-follicle granulosa cell PAPP-A gene expression. The Ct and  $\Delta$ Ct values are summarized in table 3.

**Experiment 10: Effect of insulin, LH, FSH and estradiol on PAPP-A mRNA expression in large-follicle granulosa cells in the presence of insulin.**

Large-follicle granulosa cell PAPP-A mRNA levels were not affected ( $P > 0.10$ ) by insulin, FSH, LH and combinations of FSH with 3 or 300 ng/mL of estradiol in the presence of insulin (Table 3). The Ct and  $\Delta$ Ct values are summarized in table 3.

**Experiment 11: Effect of insulin, LH and estradiol on PAPP-A mRNA in large-follicle theca cells.**

Treatments with 100 ng/mL of insulin decreased ( $P < 0.05$ ) PAPP-A mRNA levels in large-follicle theca cells, whereas 30 ng/mL of LH or 500 ng/mL of estradiol alone did not change ( $P > 0.10$ ) PAPP-A gene expression in these cells. LH did not affect ( $P > 0.10$ ) the insulin-induced decrease in PAPP-A mRNA abundance whereas estradiol induced a further decrease ( $P < 0.05$ ) in insulin-suppressed PAPP-A gene expression in large-follicle theca cells as compared to estradiol or insulin alone (Figure 5). The Ct and  $\Delta$ Ct values are summarized in table 3.

**Experiment 12: Effect of cortisol on large-follicle theca cell PAPP-A mRNA.**

Treatments with 3 or 30 ng/mL of cortisol combined with insulin did not affect ( $P > 0.10$ ) PAPP-A gene expression in large-follicle theca cells. The Ct and  $\Delta$ Ct values are summarized in table 4.

## 5. Discussion

The current study is the first to detect the presence of PAPP-A mRNA in bovine theca cells as well as to characterize the hormonal control of PAPP-A gene expression in small (1-5 mm) and large (>7.9 mm) follicle granulosa and large-follicle theca cells in cattle. In particular, this study is the first to evaluate the effects of insulin, IGF-I and -II, the gonadotropins FSH and LH, the steroids estradiol and cortisol, and the adipose hormone leptin on PAPP-A mRNA expression in granulosa and theca cells in vitro. Investigation of the hormonal control of PAPP-A mRNA in granulosa and theca cells may reveal possible regulatory mechanisms involved in the control of the PAPP-A/IGF system.

In the present study, insulin with or without LH decreased large-follicle thecal PAPP-A mRNA by 2-fold, whereas insulin did not affect granulosa cell PAPP-A mRNA. Consistent with our findings, IGFBP-4 protease (i.e. PAPP-A activity) was not affected by insulin in cultured human granulosa cells (Iwashita et al., 1998). Using bovine granulosa cells, insulin did not affect IGFBP-4 but decreased IGFBP-5 production whereas insulin increased IGFBP-5 without affecting IGFBP-4 production by bovine theca cells (Chamberlain and Spicer, 2001). Insulin decreased IGFBP-4 mRNA without affecting IGFBP-5 mRNA in theca cells and did not affect IGFBP-4 or -5 mRNA in granulosa cells (Voge et al., 2004 a). Thus, insulin appeared to have differential effects on PAPP-A production and activity within the two cell layers of bovine follicles, increasing (or not affecting) granulosa cell PAPP-A activity while decreasing thecal PAPP-A activity.

Estradiol alone decreased theca cell PAPP-A mRNA levels, and amplified the insulin-induced inhibition of PAPP-A mRNA expression by 2.5-fold, whereas estradiol did not affect granulosa cell PAPP-A mRNA levels in the present study. Similarly, PAPP-A mRNA levels in granulosa cells did not differ between estrogen dominant and subordinate follicle, 24 or 48 h after PGF2 $\alpha$  injections in cattle (Santiago et al., 2005) or between ovulatory and non-ovulatory dominant follicles (White, 2003). Also, Santiago et al. (2005) found no significant correlation between granulosa cell PAPP-A mRNA and estradiol measured in follicular fluid of dominant and subordinate follicles. Similar to our studies, differences in PAPP-A mRNA levels were not detected in small and large healthy bovine or porcine follicle granulosa cells, although a positive correlation between PAPP-A mRNA levels and aromatase mRNA in porcine and bovine granulosa cells were observed (Mazerbourg et al., 2001). Granulosa cell secretion of IGFBP-4 protein, one of the PAPP-A substrates, was decreased by estradiol (Spicer et al., 2001), whereas IGFBP-4 mRNA in granulosa cells was not affected by estradiol (Voge et al., 2004 a). Similarly, estradiol decreases IGFBP-5 protein production (Spicer and Chamberlain, 2002) while having no effect on IGFBP-5 mRNA abundance in small follicle granulosa cells (Voge et al., 2004a). Thus, decreases in IGFBP-4 and -5 in the estrogen dominant follicle may be due to decreases in the IGFBP-4 and -5 mRNA as well as their proteolysis. Estradiol-induced changes in PAPP-A mRNA in theca cells may be part of an intrafollicular negative feedback system to limit PAPP-A production and thus limit bioavailable IGFs within the theca layer in order to prevent excessive androgen production.

Our research found that PAPP-A gene expression was not affected by gonadotropins in granulosa or theca cells, and FSH at either a low (3 ng/mL) or high (30

ng/mL) doses did not change PAPP-A mRNA levels in either small- or large-follicle granulosa cells. In contrast, PAPP-A mRNA levels in cultured granulosa cells of late pre-antral rat follicles were increased by FSH within 12 and 24 h (Matsui et al., 2004), and PAPP-A mRNA levels in whole ovarian extracts increased between 4 and 36 h post-PMSG treatment in mice (Hourvitz et al., 2002). However, FSH induced IGFBP-4 degradation (i.e., PAPP-A activity) in rat and human cultured granulosa cells (Liu et al., 1993; Shimasaki et al., 1990; Iwashita et al., 1998), but whether this was a direct effect on PAPP-A gene expression was not evaluated. FSH did not change basal or insulin-inhibited IGFBP-5 production by bovine granulosa cells (Chamberlain and Spicer, 2001) or affect IGFBP-4 and -5 mRNA abundance in bovine granulosa cells (Voge et al., 2004 a; Voge et al., 2004 b), suggesting that PAPP-A gene expression is not FSH dependant in cattle. These differences in the response of granulosa cell PAPP-A mRNA level to FSH may be species or culture condition specific. How FSH affects PAPP-A proteolytic activity without altering its production will require further elucidation.

Consistent with the present finding that LH had no effect on PAPP-A mRNA levels in theca or granulosa cells, Chamberlain and Spicer (2001) found no differences in production of IGFBP-2/-5 and -4 proteins by LH-treated theca cells, and Voge et al. (2004 b) found no effect of LH on IGFBP-2, -4 or -5 mRNA levels in granulosa and theca cells. To the best of our knowledge, no previous reports were conducted that evaluated the effects of LH on PAPP-A mRNA abundance in granulosa or theca cells of any species. Previously, Conover et al. (2001) reported that hCG had no effect on PAPP-A protein levels secreted by human granulosa cells and agrees with the present study. In contrast, Hourvitz et al. (2002) showed that hCG treatment in vivo increased whole

ovarian PAPP-A mRNA abundance, and Mazerbourg et al. (2001) found that PAPP-A mRNA was positively correlated to LH receptor mRNA in bovine and porcine granulosa cells. Collectively, lack of LH-induced increases in granulosa and thecal PAPP-A mRNA and protein production during final follicular growth may allow for a constant supply of bioavailable IGF-I and -II for follicular cell differentiation.

From the metabolic hormones shown to affect reproduction, we tested leptin and cortisol since leptin, an adipose hormone, influences the ovary by antagonizing insulin-induced steroidogenesis (Spicer and Francisco, 1997), while cortisol affects thecal androstenedione production, increasing theca cell numbers and protecting the follicle from atresia (Spicer and Chamberlain, 1998). Neither leptin nor cortisol affected PAPP-A gene expression in granulosa or theca cells of the present study. Previously, Voge et al. (2004 b) showed that leptin and cortisol did not affect IGFBP-4 and -5 mRNA in cultured granulosa or theca cells using quantitative RT-PCR, whereas Chamberlain and Spicer (2001) showed that cortisol decreased IGFBP-4 and -5 production by granulosa cells but had no effect in thecal cells. Bischof et al. (1986) reported that cortisol had no effect on PAPP-A secretion by human decidual explants, and Conover et al. (2004) and Jia and Heersche (2005) reported that dexamethasone had no effect on PAPP-A mRNA in rat bone cells. Collectively, these results indicate that cortisol may increase PAPP-A activity in bovine granulosa cells without affecting PAPP-A gene expression.

When compared across experiments of the current study and cell types, PAPP-A mRNA was 5.5-fold lower in large-follicle theca cells than in either small or large-follicle granulosa cells, suggesting that the granulosa cells are the main site of follicular PAPP-A production. A similar conclusion was made from experiments using in situ hybridization

in PMSG-primed mice (Hourvitz et al., 2002) and humans (Rhoton-Vlasak et al., 2003). PAPP-A mRNA is present in granulosa cells of healthy preantral to preovulatory human follicles (Hourvitz et al., 2000), and observed in rat membrane but not cumulus granulosa cells (Matsui et al., 2004). Also, PAPP-A protein was localized in luteinized granulosa and theca cells of gonadotropin-stimulated women (Sjoberg et al., 1984). How effective bovine granulosa and theca cells are in translating the PAPP-A mRNA to provide more PAPP-A protein needs further investigation.

Shown for the first time, PAPP-A mRNA expression was detected in bovine theca cells, an observation confirmed in human ovaries using in situ hybridization (Rhoton-Vlasak et al., 2003). In the present experiment, IGF-II did not affect PAPP-A mRNA in granulosa cells, whereas in large-follicle theca cells, IGF-II stimulated an increase (Experiment 2), no change (Experiment 7) or a decrease (Experiment 5) in PAPP-A mRNA levels. When averaged across these 3 sets of experiments, IGF-II did not significantly affect PAPP-A mRNA levels in large-follicle theca cells (data not shown). Consistent with our results, Santiago et al. (2005) found no correlation between granulosa PAPP-A mRNA and free IGF-I or total IGF-II in dominant and subordinate follicles collected during the follicular phase, and Conover et al. (2001) found that levels of PAPP-A protein secreted by human granulosa cells were not affected by 100 ng/mL of IGF-II, FSH or hCG. In addition, IGF-I and -II are needed for PAPP-A proteolytic activity on IGFBP-4 (but not IGFBP-5) to be fully expressed via IGF binding to IGFBP-4 and making it more susceptible to PAPP-A proteolysis. Furthermore, major basic protein precursor protein (proMBP) binds reversibly to PAPP-A (Overgaard et al., 2000; Chen et al., 2002; Glerup et al., 2005), an association that leads to the inactivation of the PAPP-A

proteolytic activity and thus may modify PAPP-A's role in follicular selection. In ovarian tumors, increased IGF-II mRNA production was accompanied with the lowest observed PAPP-A mRNA and increased proMBP mRNA (Kalli et al., 2004) whereas proMBP cDNA-transfected cells inhibited IGFBP-4 proteolytic activity in fibroblasts without affecting PAPP-A protein synthesis in these cells (Chen et al., 2002). Thus, further characterization of the IGF-II effect on theca cells and its relation to the proMBP mRNA and its hormonal control should be elucidated.

The current study revealed that in small- and large-follicle granulosa cells, PAPP-A mRNA levels were not significantly affected by any of the hormones tested including insulin, IGFs, gonadotropins, estradiol or leptin. On the other hand, thecal PAPP-A mRNA abundance was decreased by insulin and estradiol, and estradiol worked in concert with insulin to create a greater negative feedback loop on PAPP-A mRNA abundance in theca cells. Granulosa cell-derived estradiol may act as a paracrine negative feedback regulator to suppress PAPP-A mRNA production in theca cells, and therefore less IGFBP-4 and/or -5 proteolysis which would allow for less bioavailable IGFs for proliferation and steroid production by theca cells. With further development of bovine-specific PAPP-A reagents, the relationship between PAPP-A mRNA and PAPP-A protein levels and activity can be further investigated and elucidated. Also, the post-translational control of PAPP-A proteolytic activity in bovine follicles will also require further study.

**Figure 1:** Effect of time and IGF-I on PAPP-A gene expression in large-follicle (> 7.9 mm) granulosa cells in experiment 1. Cells were cultured for 48 h as described in Section 2, and then treated for 0, 12 or 24 h in serum-free media with: control (no hormones) or 100 ng/mL of IGF-I.



## Large-Follicle Granulosa Cells

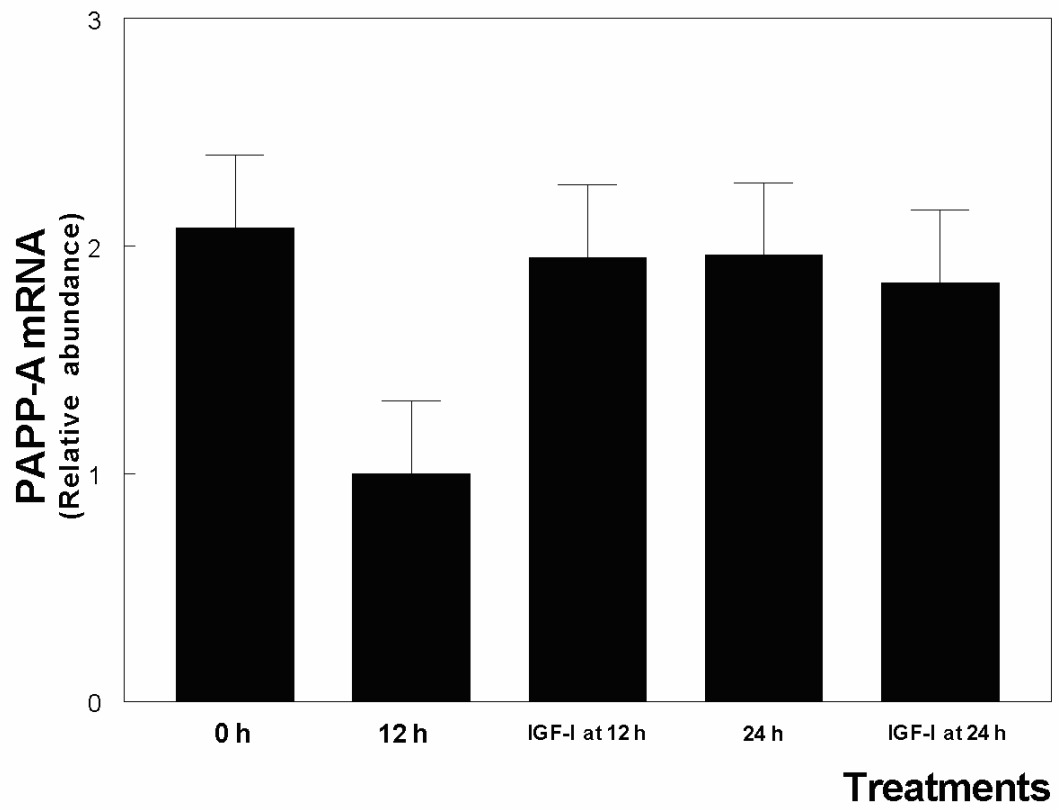


FIGURE 1

**Figure 2:** Effect of time and IGF-II on PAPP-A gene expression in large (>7.9 mm) theca cells in experiment 2. Cells were cultured for 48 h as described in Section 2, and then treated for 0, 12 or 24 h in serum-free media with: control (no hormones) or 100 ng/mL of IGF-II. Means without a common letter differ ( $P < 0.05$ ).

## Large-Follicle Theca Cells

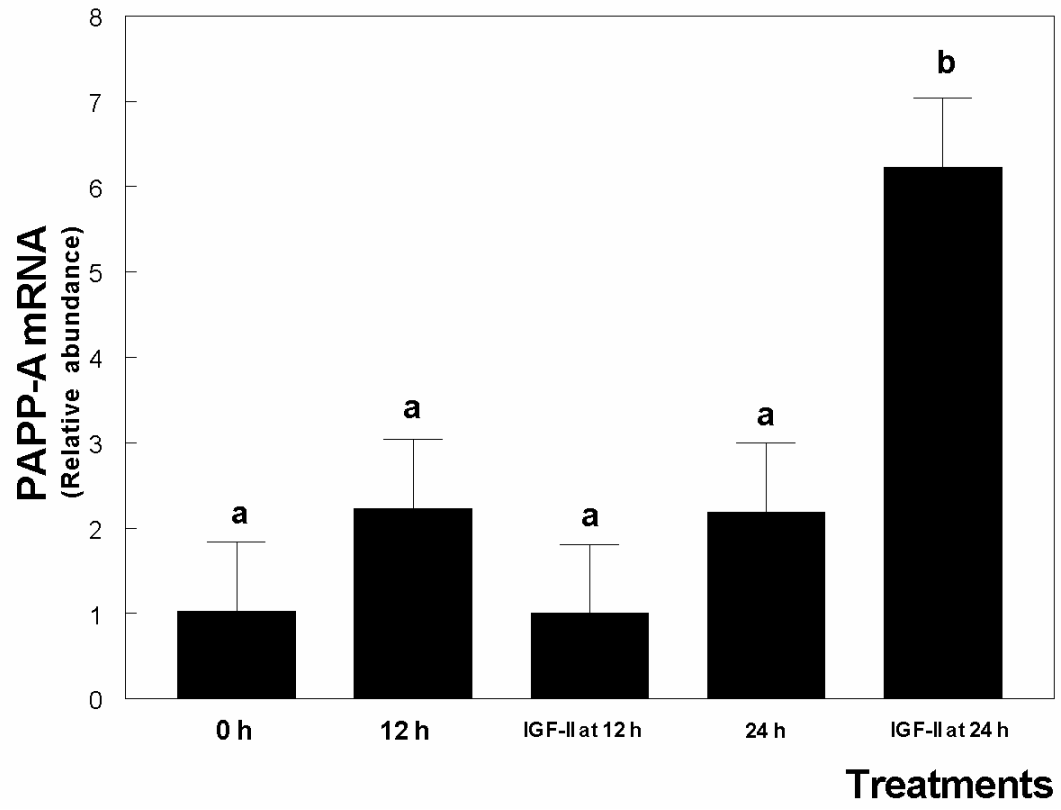


FIGURE 2

**Figure 3:** Effect of IGF-I and IGF-II on PAPP-A mRNA in large-follicle (>7.9 mm) theca cells in experiment 5. Cells were cultured for 48 h as described in Section 2, and then treated for 24 h in serum-free media with : control (no hormones), 100 ng/mL of IGF-I or 100 ng/mL of IGF-II. Means without a common letter differ ( $P < 0.05$ ).

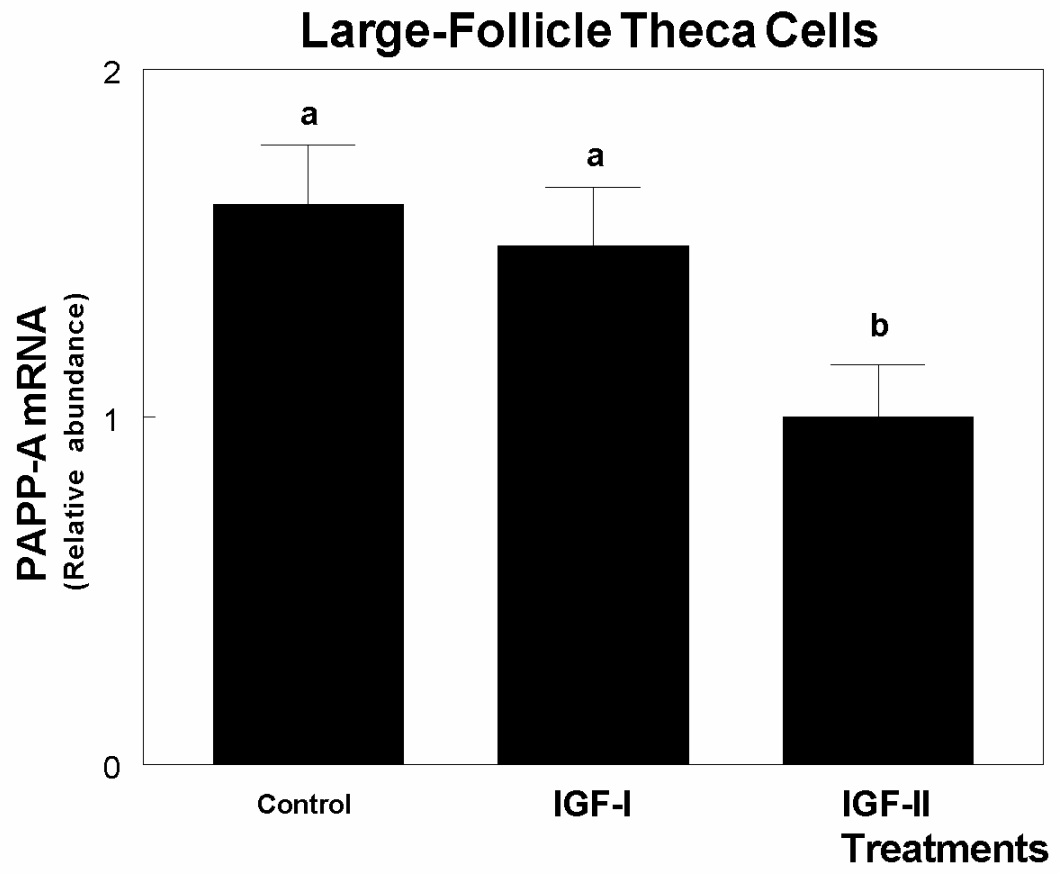


FIGURE 3

**Figure 4:** Effect of IGF-II and estradiol on PAPP-A mRNA in large-follicle (>7.9 mm) theca cells in experiment 7. Cells were cultured for 48 h as described in Section 2, and then treated for 24 h in serum-free media with: control (no hormones), 100 ng/mL of IGF-II, 3 ng/mL of estradiol (E), IGF-II plus 3 ng/mL of E, 300 ng/mL of E, or IGF-II plus 300 ng/mL of E. Means without a common letter differ ( $P < 0.05$ ).

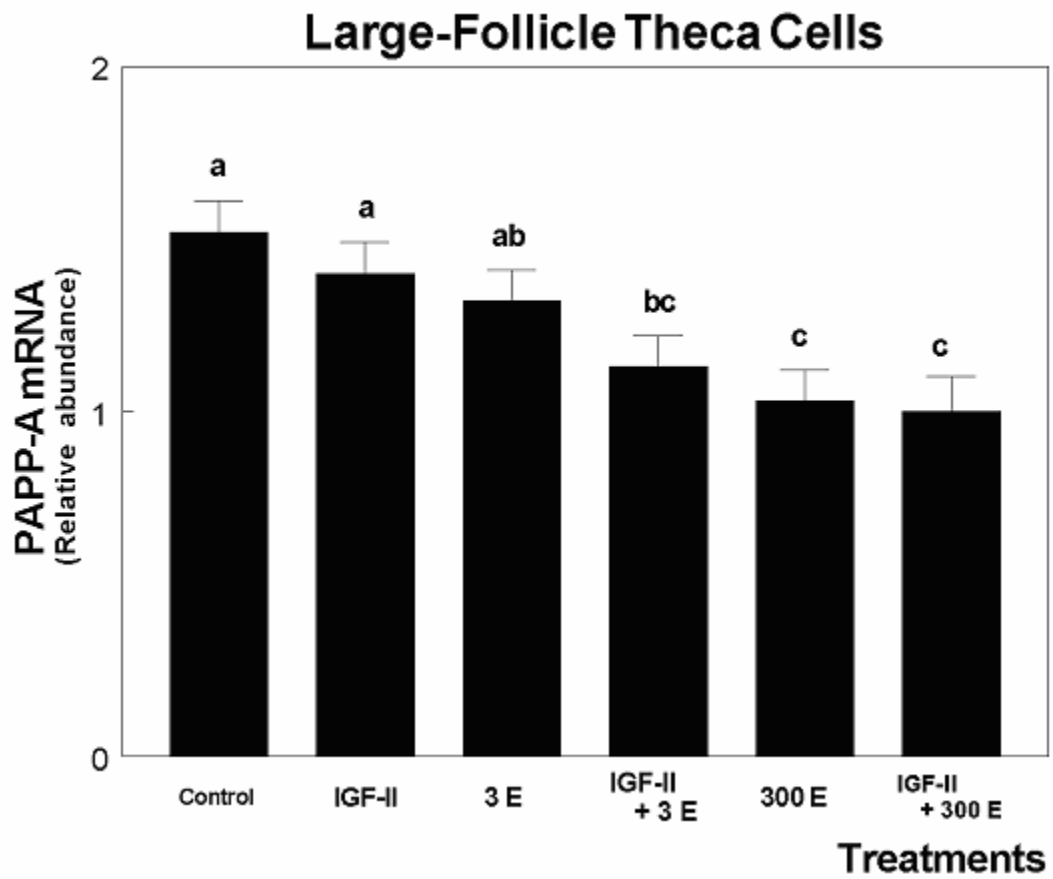


FIGURE 4

**Figure 5:** Effect of insulin, LH and estradiol on PAPP-A mRNA in large-follicle (>7.9 mm) theca cells in experiment 11. Cells were cultured for 48 h as described in Section 2, and then treated for 24 h in serum-free media with: control (no hormones), 30 ng/mL of LH, 100 ng/mL of insulin (INS), 500 ng/mL of E, 30 ng/mL of LH plus 500 ng/mL of E or 500 ng/mL of E plus 100 ng/mL of insulin. Means without a common letter differ ( $P < 0.05$ ).



## Large-Follicle Theca Cells

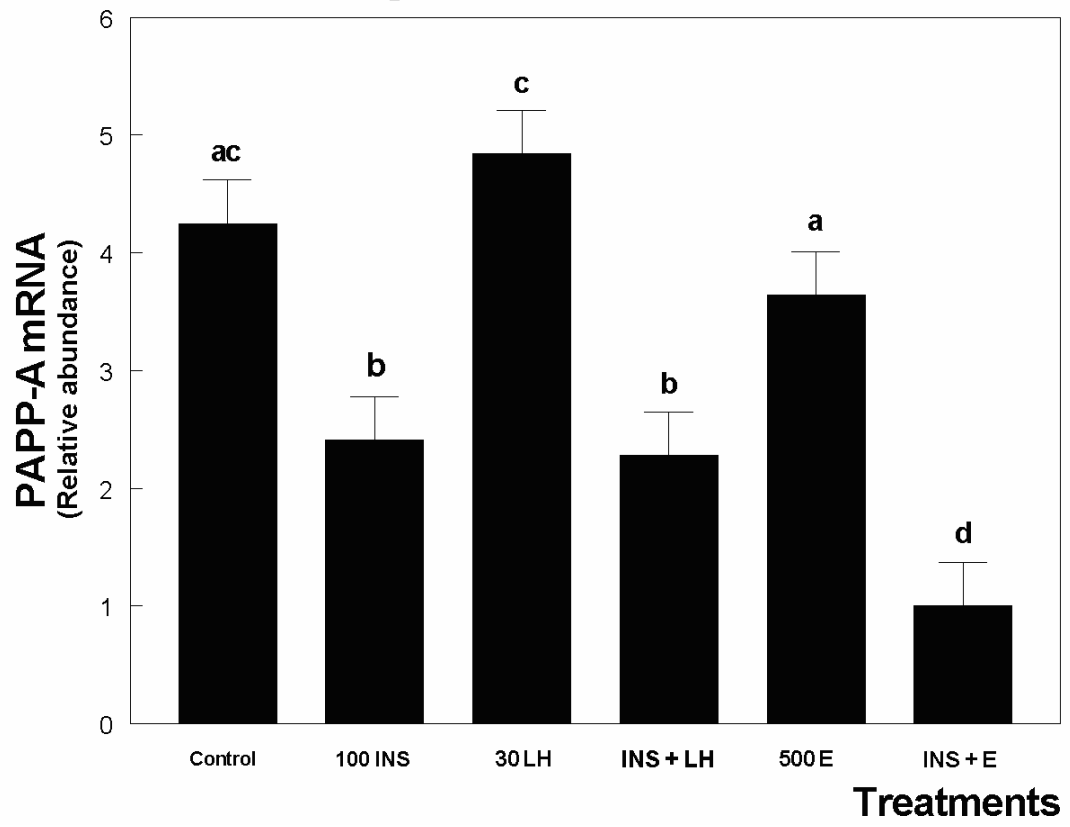


FIGURE 5

**Table 1:** Quantitative real-time PCR analysis of PAPP-A mRNA expression in large-follicle granulosa (Experiment 1) and theca (Experiments 2) cells treated with no hormones (0 h, 12 h and 24 h), IGF-I or IGF-II and RNA collected at 0 h, 12 h or 24 h after treatment application.

Cell Type	Treatment	PAPP-A Ct	18 S Ct	$\Delta$ Ct
<b>Experiment 1</b>				
<b>LG-GC</b>				
	<b>0 h</b>	20.45	19.30	1.15
	<b>12 h</b>	21.18	18.99	2.20
	<b>12h + IGF-I</b>	20.59	19.36	1.23
	<b>24 h</b>	20.82	19.47	1.35
	<b>24 h + IGF-I</b>	20.84	19.50	1.34
	<b>S.E.M</b>	0.16	0.20	0.26
<b>Experiment 2</b>				
<b>LG-TC</b>				
	<b>0 h</b>	21.61	16.84	4.77 <sup>a</sup>
	<b>12 h</b>	22.12	18.36	3.76 <sup>a</sup>
	<b>12h + IGF-II</b>	22.48	17.91	4.58 <sup>a</sup>
	<b>24 h</b>	22.51	18.38	4.13 <sup>a</sup>
	<b>24 h + IGF-II</b>	22.95	20.97	1.99 <sup>b</sup>
	<b>S.E.M</b>	0.14	0.52	0.48

Within a specific experiment,  $\Delta$ Ct values without a common letter (a, b, c) differ ( $P < 0.05$ ).

**Table 2:** Quantitative real-time PCR of PAPP-A mRNA expression in small- (Experiments 1 and 6) and large- (Experiment 4) follicle granulosa and theca (Experiments 5 and 7) cells treated with either no hormones (Control), IGF-I, IGF-II, FSH, leptin and (or) estradiol (E).

Cell Type	Treatment	PAPP-A Ct	18 S Ct	$\Delta$ Ct
<b>Experiment 3</b>				
<b>SM-GC</b>				
	Control	21.64	20.88	0.77
	100 IGF-I	20.19	18.99	1.20
	100 IGF-II	20.30	19.21	1.08
	S.E.M	0.27	0.48	0.25
<b>Experiment 4</b>				
<b>LG-GC</b>				
	Control	24.90	21.23	3.67
	3 IGF-I	23.42	18.97	4.45
	100 IGF-I	23.77	19.64	4.13
	100 IGF-II	23.12	19.48	3.64
	30 FSH	24.35	20.09	4.25
	100 Leptin	24.13	19.93	4.20
	S.E.M	0.35	0.34	0.26
<b>Experiment 5</b>				
<b>LG-TC</b>				
	Control	25.93	23.46	2.48 <sup>a</sup>
	100 IGF-I	24.77	21.84	2.92 <sup>a</sup>
	100 IGF-II	24.69	21.46	3.23 <sup>b</sup>
	S.E.M	0.30	0.36	0.17
<b>Experiment 6</b>				
<b>SM-GC</b>				
	Control	24.46	23.31	1.15
	100 IGF-I	24.77	23.47	1.31
	30 FSH	24.85	23.54	1.31
	IGF-I + FSH	24.83	23.58	1.26
	S.E.M	0.14	0.70	0.34
<b>Experiment 7</b>				
<b>LG-TC</b>				
	Control	22.39	17.78	4.62 <sup>a</sup>
	100 IGF-II	22.68	17.99	4.69 <sup>a</sup>
	3 E	22.58	17.79	4.79 <sup>ab</sup>
	IGF-II + 3 E	23.07	18.07	5.00 <sup>bc</sup>
	300 E	23.08	17.91	5.17 <sup>c</sup>
	IGF-II + 300 E	23.04	17.82	5.22 <sup>c</sup>
	S.E.M	0.31	0.32	0.10

Within a specific experiment,  $\Delta$ Ct values without a common letter (a, b, c) differ ( $P < 0.05$ ).

**Table 3:** Quantitative real-time PCR of PAPP-A mRNA expression in small- (Experiments 8 and 9) and large- (Experiment 10) follicle granulosa and theca (Experiments 11) cells treated with either no hormones (Control), insulin (INS), FSH, LH and (or) estradiol (E).

Cell type	Treatment	PAPP-A Ct	18 S Ct	$\Delta$ Ct	
<b>Experiment 8</b>					
SM-GC					
	Control	22.88	15.22	7.65	
	300 E	22.29	15.50	6.79	
	30 FSH	23.05	15.50	7.54	
	E + FSH	22.92	15.55	7.37	
	30 LH	22.99	15.58	7.41	
	E+LH	24.11	15.48	8.64	
	S.E.M	0.60	0.22	0.59	
<b>Experiment 9</b>					
SM-GC					
	100 INS	22.93	20.67	2.26	
	INS + 30 LH	22.87	21.11	1.75	
	INS + 3 FSH	22.59	20.56	2.03	
	INS + 30 FSH	22.39	20.75	1.63	
	INS + 30 FSH + 3 E	22.59	20.90	1.69	
	INS + 30 FSH + 300 E	22.59	20.80	1.79	
	S.E.M	0.23	0.24	0.25	
<b>Experiment 10</b>					
LG-GC					
	Control	23.28	17.67	4.11	
	INS	22.54	17.7	4.17	
	INS + LH	23.76	18.16	3.18	
	INS + FSH	22.49	17.51	4.09	
	INS + FSH + 3 E	23.06	18.06	4.17	
	INS + FSH + 300 E	21.79	17.04	3.80	
	S.E.M	0.65	0.23	0.61	
<b>Experiment 11</b>					
LG-TC					
	Control	21.69	18.68	3.01	ac
	100 INS	22.40	18.57	3.84	b
	30 LH	22.21	19.38	2.82	c
	INS + LH	22.23	18.27	3.97	b
	500 E	22.37	19.09	3.29	a
	INS + E	22.64	17.37	5.27	d
	S.E.M	0.24	0.25	0.16	

Within a specific experiment,  $\Delta$ Ct values without a common letter (a, b, c) differ ( $P < 0.05$ ).

**Table 4:** Quantitative real-time PCR of PAPP-A mRNA expression in large-follicle theca cells (Experiments 12) with insulin (INS) and cortisol (CORT).

<b>Cell type</b>	<b>Treatment</b>	<b>PAPP-A Ct</b>	<b>18 S Ct</b>	<b><math>\Delta</math>Ct</b>
<b>Experiment 12</b>				
<b>LG-TC</b>				
	<b>INS</b>	26.6	21.11	5.15
	<b>INS + 3 CORT</b>	26.10	20.20	4.90
	<b>INS + 30 CORT</b>	26.43	21.12	5.31
	<b>S.E.M</b>	0.64	0.60	0.39

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#### IV. SUMMARY AND CONCLUSION

A cascade of autocrine and paracrine hormonal events control follicular growth and development. The IGF/IGFBP system showed to be a major controller of the IGF availability for cell mitogenesis and steroidogenesis. PAPP-A is responsible for IGFBP-4 proteolysis, an event that marks follicular selection toward either atresia or dominance. In this study, we characterized ovarian cell PAPP-A gene expression in response to various reproductive and metabolic hormones, and found that: 1) IGF-I or -II did not alter PAPP-A mRNA levels in granulosa and theca cells; 2) insulin, estradiol, LH, FSH and leptin did not affect PAPP-A mRNA levels in granulosa cells of small and large follicles; 3) insulin with or without LH decreased large-follicle theca PAPP-A mRNA abundance by 2-fold; and 4) estradiol alone decreased thecal PAPP-A mRNA levels, and amplified the insulin-induced inhibition of thecal PAPP-A mRNA expression.

In conclusion, granulosa cells contain more PAPP-A mRNA than theca cells but are not regulated by the main reproductive hormones, whereas theca cells production of PAPP-A mRNA is regulated negatively by insulin and estradiol. Insulin and estradiol work in concert to decrease thecal PAPP-A mRNA. The selective decrease in thecal PAPP-A gene expression induced by estradiol may lead to a decrease in proteolysis of IGFBP-4/-5 and thus less bioavailable IGFs within the theca layer. We hypothesize that

estradiol acts as a negative paracrine feedback regulator to prevent excessive IGF-I – induced androgen production, and hence prevent excessive estradiol production by granulosa cells. This control of thecal PAPP-A production likely maintains desirable levels of IGFBP-4 and -5 and subsequently free IGF-I /-II within the follicle during follicular development. The current study elucidates the hormonal control of PAPP-A gene expression in granulosa and theca cells, however further research is needed to determine the post-transcriptional regulators of PAPP-A protein and proteolytic activity and their impact on follicular development and selection.



## Vita

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Title of Study: REAL-TIME RT-PCR QUANTIFICATION OF PREGNANCY-

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Scope of Study: Ovarian follicular growth and dominance is controlled by a series of hormonal events including a decrease in intrafollicular IGF-binding proteins -2, -4 and -5 levels. Proteolytic enzymes such as pregnancy-associated plasma protein-A (PAPP-A) degrade IGF-BPs and increase availability of IGF-I and -II during follicular development. The objective of this study was to determine the effect of IGF-I, IGF-II, insulin (INS), LH, FSH, estradiol (E), leptin or cortisol on ovarian PAPP-A mRNA levels. Granulosa (GC) from small (SM) (<5 mm) and large (LG) (>7.9 mm) follicles as well as theca cells (TC) from LG follicles were collected from bovine ovaries and cultured for 48 h in medium containing 10% FCS and then treated with various hormones in serum-free medium for an additional 12 or 24 h. LG-GC and LG-TC were cultured for either 12 or 24 h with 0 or 100 ng/mL of IGF-I or -II (exp 1 and 2). In all other experiments, SM-GC LG-GC and LG-TC were treated with various combinations of IGF-I, IGF-II, FSH, LH, E, INS, leptin and (or) cortisol for 24 h (exp 3 through 12). PAPP-A mRNA expression levels quantified using quantitative real-time RT-PCR.

Findings and Conclusions: Although more PAPP-A mRNA was produced by SM-GC and LG-GC than LG-TC, none of the treatments significantly affected ( $P>0.10$ ) PAPP-A gene expression in SM-GC and LG-GC. In LG-TC, IGF-I, IGF-II, LH or CORT did not affect ( $P>0.10$ ) PAPP-A mRNA levels, whereas INS with or without LH decreased ( $P<0.05$ ) PAPP-A mRNA by 2-fold; E alone decreased PAPP-A mRNA levels, and amplified the insulin-induced inhibition of PAPP-A mRNA expression. We conclude that PAPP-A gene expression is differentially regulated in granulosa and theca cells. Insulin alone and in combination with E decreased the PAPP-A mRNA levels which would likely reduce the bioavailable IGFs in the theca layer during growth and selection of follicles. Thus, E and INS may act as negative feedback regulators to prevent excessive IGF-I – induced androgen production, and hence prevent excessive estradiol production by granulosa cells via decreased thecal PAPP-A production and therefore maintain desirable levels of IGF-BP-4 and -5 and subsequently free IGF-I /-II within the follicle during follicular development.

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