Insulin and IGF-I are necessary for FSH-induced cytochrome P450 aromatase but not cytochrome P450 side-chain cleavage gene expression in oestrogenic bovine granulosa cells in vitro

J M Silva and C A Price
Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, 3200 Sicotte, St-Hyacinthe, Québec J2S 7C6, Canada

(Requests for offprints should be addressed to C A Price; Email: christopher.price@umontreal.ca)
(J M Silva is now at Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Zacatecas, Zacatecas, Mexico)

Abstract
The earliest biochemical indicators of ovarian follicle deviation in cattle include lower oestradiol and free IGF concentrations in subordinate compared with dominant follicles. We determined if decreases in FSH, IGF-I or insulin cause decreased P450 aromatase (P450arom) or P450 cholesterol side-chain cleavage (P450scc) mRNA expression in oestrogenic bovine granulosa cells in vitro. In the first experiment, cells obtained from small follicles (2–5 mm diameter) were cultured in serum-free medium supplemented with physiological concentrations of FSH, IGF-I and insulin for 4 days. A decrease in specific hormone concentration was produced by replacing 70% of spent medium with medium devoid of FSH, insulin, or insulin and IGF-I on day 4 and again on day 5 of culture. Cultures were terminated on day 7. A reduction in FSH concentrations during the last 3 days of culture decreased P450arom and P450scc mRNA levels. A reduction in insulin reduced P450arom but not P450scc mRNA levels, and a reduction of both insulin and IGF-I concentrations further decreased P450arom mRNA levels and decreased P450scc mRNA levels. In a second experiment, cells obtained from small follicles (2–5 mm diameter) were cultured with insulin (100 ng/ml) without FSH for 4 days, and then insulin was withdrawn from the culture and FSH added for a further 3 days. The withdrawal of insulin decreased (P<0.02) oestradiol accumulation and reduced P450arom mRNA to below detectable levels, but did not affect P450scc mRNA levels. The addition of FSH transiently increased oestradiol secretion and P450arom mRNA levels, but P450arom mRNA levels were undetectable at the end of the culture period. The addition of FSH significantly enhanced P450scc mRNA levels and progesterone accumulation. These data demonstrated that a reduction of insulin-like activity reduced aromatase gene expression in bovine follicles without necessarily affecting progesterone synthetic capability, and thus may initiate follicle regression in cattle at the time of follicle divergence.


Introduction
The major characteristic of non-atretic growing and of preovulatory ovarian follicles is the expression of cytochrome P450 aromatase (P450arom) (Xu et al. 1995, Bao et al. 1997) and, as a consequence, the ability to produce oestradiol (Badinga et al. 1992, Guilbault et al. 1993). In growing rodent follicles, oestradiol is required for the induction of luteinizing hormone (LH) receptors on granulosa cells (Segaloff et al. 1990), a prerequisite for ovulation. Inhibition of aromatase activity may induce follicle atresia (Bergfelt et al. 1999). Thus, a full understanding of the regulation of P450arom expression and activity has important implications for the control of fertility.

The primary stimulator of P450arom gene expression and enzyme activity (and follicular growth) is follicle-stimulating hormone (FSH), as demonstrated in rats, humans and ruminants (Steinkampf et al. 1987, Fitzpatrick & Richards 1991, Campbell et al. 1996, Gutiérrez et al. 1997). Follicle growth occurs in ‘waves’ in cattle, and each wave is preceded by a transient increase in plasma FSH concentrations (Adams et al. 1992, Sunderland et al. 1994). These FSH ‘transients’ are believed to stimulate the growth of a cohort of small antral follicles (reviewed by Ginther et al. 2001a). As the follicle wave progresses, one follicle emerges as the dominant follicle and the remaining follicles of the cohort regress. Regression of these subordinate follicles occurs at the end of the FSH transient, which has led to the hypothesis that the decrease in serum FSH concentrations causes atresia in subordinate follicles. Corresponding with the decline in FSH concentrations, small and medium sized follicles contain less oestradiol after compared with before dominant follicle emergence (Smith et al. 1996, Mihm et al. 1997).
Towards the end of the FSH transient, the future dominant follicle and the largest subordinate follicle are similar in size and/or growth rate (Ginther et al. 2001a), yet these two follicles diverge as FSH concentrations decline. According to the main hypotheses for follicle divergence, the future dominant follicle becomes more sensitive to FSH, and can maintain P450arom expression in a low-FSH environment (reviewed by Fortune et al. 2001). The granulosa cells then acquire LH receptors, which permit continued growth and increased steroidogenesis under the influence of LH (Ginther et al. 2001b). It is known that several growth factors stimulate bovine granulosa cell growth and steroidogenesis, including insulin-like growth factor-I (IGF-I) and insulin (Spicer & Echternkamp 1995, Gutiérrez et al. 1997, Silva & Price 2000). While concentrations of total IGF-I do not differ between dominant and subordinate follicles, free IGF-I concentrations are higher in dominant compared with the largest subordinate follicles (Beg et al. 2001). The relative amounts of free IGF-I are regulated at least in part by IGF-binding proteins (IGFBP), which are believed to sequester IGF-I (reviewed by Poretsky et al. 1999). A decrease in IGFBP could thus lead to an increase in local IGF-I bioavailability. Several studies have shown that IGFBP levels are higher in subordinate compared with dominant follicles (Echternkamp et al. 1994, Mihm et al. 1997). More recently, sampling of follicular fluid during a follicle wave has provided a detailed analysis of changes in follicular hormones during follicle deviation. By 2 days after follicle wave emergence, follicular fluid concentrations of oestradiol were higher and those of IGFBP-4 were lower in the dominant compared with the largest subordinate follicle (Mihm et al. 2000, Rivera et al. 2001).

In the model suggested by the above studies, IGF-I and FSH stimulate aromatase, and thus equip a follicle for continued growth (Fortune et al. 2001). A subsequent decrease in bioavailable IGF-I (owing to increased IGFBP) in a low-FSH environment (at the end of the FSH transient) may cause a loss of aromatase activity and initiate regression of future subordinate follicles. While it has been shown that IGF-I and/or insulin stimulate oestradiol secretion in several cell culture systems (Willis et al. 1996, Gutiérrez et al. 1997, Silva & Price 2000), it is not known whether the decrease in free IGF-I observed in vivo is a cause or a result of early follicle regression and decrease in aromatase activity. This is underscored by the observation that the divergence in oestradiol concentrations between dominant and subordinate follicles occurs before that in IGFBP concentrations (Austin et al. 2001).

The objectives of the present study were to test the hypothesis that a reduction in IGF-I and/or insulin concentrations decreases P450arom mRNA expression and enzyme activity in oestrogenic bovine granulosa cells in vitro, and thus to identify the nature of the causal relationship between these hormones. We also studied another major steroidogenic enzyme, cytochrome P450 cholesterol side-chain cleavage (P450sc), which is responsive to FSH but not insulin in the cell model used herein (Silva & Price 2000). As exogenous FSH is known to prevent atresia in subordinate follicles in ruminants (Adams et al. 1993, Jablonska-Shari et al. 1996), we also tested the hypothesis that increased FSH can prevent a decrease in P450arom mRNA expression caused by a reduction of IGF-I/insulin.

Materials and Methods

Cell culture

The cell culture system was based on that described previously (Gutiérrez et al. 1997, Silva & Price 2000). All materials were obtained from Gibco BRL Canada (Burlington, ON, Canada) unless otherwise stated. Bovine ovaries were collected from adult cows, irrespective of the stage of the oestrous cycle, at a local abattoir and transported to the laboratory in culture medium M199 containing 25 mM Hepes, penicilllin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). Follicles were dissected free of surrounding tissue, and small follicles (2–5 mm diameter) were bisected into culture medium at 37 °C. Granulosa cells were recovered by drawing the follicle walls repeatedly in and out of a polyethylene disposable transfer pipette. The suspension was filtered through a steel sieve (80 mesh; Sigma Chemical Co., St Louis, MO, USA), and granulosa cells were isolated by centrifugation at 800 g for 5 min. After washing three times in M199 containing 25 mM Hepes, penicillin (100 IU/ml) and streptomycin (100 µg/ml), the cells were resuspended in culture medium, and cell viability was estimated to be approximately 40% by Trypan blue exclusion, as previously observed (Silva & Price 2000).

Cells were seeded into 24-well tissue culture plates (Falcon; Becton Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 106 viable cells in 1 ml α-minimum essential medium with 1-glutamine containing sodium bicarbonate (10 mM), Hepes (20 mM), protease-free bovine serum albumin (0.1%), sodium selenite (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (10−7 M), insulin (10 or 100 ng/ml), human recombinant IGF-I (10 ng/ml), non-essential amino acid mix (1:1 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cultures were maintained at 37 °C in 5% CO2 for 7 days, with 700 µl medium being replaced on days 2 and 4. Bovine FSH (USDA-bFSH-I-1 (AFP-5310B); biological potency 1693 U/mg relative to 2nd IRP-HMG) (NIADDK, Torrance, CA, USA) was used at the doses described in each experiment.

Experimental treatments

In experiment 1, cells were cultured as described above with FSH (1 ng/ml) and insulin (10 ng/ml). On the afternoon of day 4 of culture, the cells were subjected to a..
reduction in FSH, IGF-I and/or insulin concentrations by replacing 700 µl of spent culture medium with medium devoid of FSH, IGF-I and/or insulin. On the morning of the following day (day 5), a further medium change was performed and cells were cultured for a further 2 days. This treatment regime was designed to reduce IGF-I and insulin concentrations from 10 ng/ml to 3 ng/ml for 12 h, and then to 0·9 ng/ml for the remaining 48 h (calculated concentrations). These concentrations are at the low end of the physiological range in cattle (Richards et al. 1989, Beg et al. 2001). Control cultures were maintained in FSH-, IGF-I- and insulin-supplemented medium.

In experiment 2, cells were cultured in the presence of a high dose of insulin (100 ng/ml) but without FSH. On the afternoon of day 4 of culture, the cells were subjected to a reduction in IGF-I and insulin concentrations by replacing 700 µl of spent culture medium with medium devoid of IGF-I and insulin, but supplemented with 0, 1, 10 or 100 ng/ml FSH. On the morning of the following day (day 5), a further medium change was performed and cells were cultured for a further 2 days. Control cultures were maintained in IGF-I- and insulin-supplemented medium in the absence of FSH.

Granulosa cells were recovered for the extraction of nucleic acids, and the culture medium frozen at −20 °C for oestradiol and progesterone assays. Twelve wells were pooled per treatment, and all treatments were applied to the same pool of cells within replicate. Cultures were repeated with three independent pools of cells, and experiments 1 and 2 were performed with different pools of cells. The same wells were used for extraction of total RNA and DNA using Trizol (Gibco BRL) according to the manufacturer’s instructions. Total DNA was quantified by measuring fluorescence in the presence of Hoechst 33258 (Labarca & Paigen 1980) and compared with a calf thymus DNA standard (Boehringer Mannheim, Laval, PQ, Canada).

Hybridization

The relative abundance of mRNA was determined by Northern hybridization. Electrophoresis of 15 µg total RNA was performed through a 1% denaturing formaldehyde–agarose gel followed by overnight capillary

![Image of Northern blot and graph](image)

Figure 1 Oestradiol accumulation and P450arom mRNA abundance in bovine granulosa cells after selective hormone reduction. Cells were cultured for 4 days with insulin, IGF-I and FSH (10, 10 and 1 ng/ml respectively). Hormone reduction was accomplished by replacement of 70% of spent medium with medium devoid of the indicated hormones on days 4 and 5 of culture. Cells were harvested on day 7. Values are least-squares means ± S.E.M. and were obtained from three replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and steroids are expressed relative to total cell DNA. A representative Northern blot is shown. Different letters above bars denote significant differences between treatment groups (P<0·01). *P<0·01, different from all other groups.
transfer onto a nylon membrane (Hybond-N; Amersham, Oakville, ON, Canada). Membranes were UV cross-linked in a commercial UV chamber (Bio Rad, Mississauga, ON, Canada) and incubated for 2 h in prehybridization solution, containing 10% dextran sulfate, 5 strength saline–sodium phosphate–EDTA buffer (SSPE), 5 strength Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS) and herring sperm DNA (200 mg/ml).

The bovine P450arom cDNA probe was prepared in our laboratory (Soumano et al. 1996), and encompasses the region coding for the entire heme-binding and I-helix regions. The probe is specific for oestrogen-secreting tissues, and hybridizes to three bands of follicular total RNA at 6.5, 3.4 and 1.8 kb, of which only the 6.5 kb transcript is polyadenylated. The bovine P450scc cDNA was a gift from Dr M R Waterman (Vanderbilt University School of Medicine, Nashville, TN, USA), and is a 1.7 kb cDNA containing the complete coding sequence (John et al. 1984), which hybridizes to a single band of 2 kb in bovine follicles (Soumano & Price 1997).

Probes were labelled with [α-32P]dCTP by random primer extension using a kit (Boehringer Mannheim) to a specific activity of 1.5–3.0 × 107 d.p.m./mg, and purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega, Montréal, PQ, Canada). Hybridization to the membranes was performed overnight at 65°C. After hybridization, membranes were washed in 2× SSPE–0.1% SDS twice at room temperature (15 min each), and twice at 65°C (15 min each). Membranes were hybridized to a labelled human 28S ribosomal cDNA probe (Gonzalez et al. 1985) for the standardization of RNA loading. The labelled membranes were exposed to Kodak X-Omat film at −70°C in the presence of an intensifying screen. Autoradiograms were scanned with a densitometer after 1–14 days exposure.

Steroid assays

Oestradiol was measured in conditioned medium without extraction with the assay described by Bélanger et al. (1990), with modifications (Price et al. 1995). Inter- and intra-assay coefficients of variation (CV) were 11 and 9% respectively. This antibody has 1.8% cross-reactivity with oestrone. Oestrone was measured with an antibody that had 2% cross-reactivity with oestradiol (Robertson et al. 1980 and verified by us). The inter- and intra-assay CV values were 7 and 10% respectively, and the sensitivity of these assays was equivalent to 25 and 40 pg/48 h per µg DNA for oestradiol and oestrone respectively. Progesterone was measured as described (Lafrance & Goff 1985), with inter- and intra-assay CV values of 9 and 5%, and a sensitivity equivalent to 100 pg/48 h per µg DNA.

Statistical analysis

The density of hybridization signals was corrected for RNA loading using hybridization to 28S ribosomal RNA,

Figure 2 Progesterone accumulation and P450scc mRNA abundance in bovine granulosa cells after selective hormone reduction. Cells were cultured for 4 days with insulin, IGF-I and FSH (10, 10 and 1 ng/ml respectively). Hormone reduction was accomplished by replacement of 70% of spent medium with medium devoid of the indicated hormones on days 4 and 5 of culture. Cells were harvested on day 7. Values are least-squares means ± S.E.M. and were obtained from three replicate experiments. Data for mRNA were corrected for loading (28S rRNA) and progesterone is expressed relative to total cell DNA. A representative Northern blot is shown. Different letters above bars denote significant differences between groups (P<0.01).
and steroid concentrations and aromatase activity are expressed relative to total DNA content of the wells. Data were transformed to logarithms when they were not normally distributed (Shapiro–Wilk test). Analysis of variance was used to test effects of treatments. Culture replicate was included as a random variable in the F-test for the effect of experiment. Differences between means were identified with the Tukey–Kramer HSD test. Correlations between steroid secretion and mRNA abundance were determined with the Pearson correlation coefficient. Analyses were performed with JMP software (SAS Institute, Cary, NC, USA). The data are presented as means ± S.E.M.

Results

In order to test the hypothesis that a reduction in IGF-I (and/or insulin) concentrations decreases P450arom mRNA expression and enzyme activity, cells were first stimulated for 4 days with FSH, IGF-I and insulin, and then subjected to reductions of either FSH, insulin and/or IGF-I. In this experiment, the withdrawal of FSH alone on day 4 decreased oestradiol accumulation and P450arom mRNA abundance \( (P<0.01; \text{ Fig. 1}) \) compared with controls. The withdrawal of insulin alone decreased P450arom mRNA levels \( (P<0.01) \) and, after the withdrawal of both insulin and IGF-I, P450arom mRNA levels were lower than after withdrawal of insulin alone \( (P<0.05; \text{ Fig. 1}) \). In neither case was oestradiol accumulation affected \( (P>0.05; \text{ Fig. 1}) \). Overall, oestradiol accumulation and P450arom mRNA levels were correlated \( (r=0.56, P=0.04, n=15) \), and this correlation improved if the divergent effects (on oestradiol and P450arom mRNA) of withdrawing insulin and IGF-I are removed from the analysis \( (r=0.75, P<0.01, n=12) \). Oestrone concentrations were consistently tenfold lower than those of oestradiol (not shown), and changed in parallel with oestradiol concentrations \( (r=0.7, P<0.01) \).

The withdrawal of FSH, and of both insulin and IGF-I, reduced P450scc mRNA levels \( (P<0.05; \text{ Fig. 2}) \), whereas progesterone accumulation was not affected by these treatments \( (P>0.05) \). Overall, the concentrations of progesterone and P450scc mRNA abundance were not correlated \( (r=0.3, P>0.1, n=15) \).

To determine whether FSH can prevent the reduction in P450arom mRNA levels following insulin and IGF-I deprivation, cells were first cultured with supraphysiological concentrations of insulin (100 ng/ml but without FSH) to induce oestradiol secretion and P450arom mRNA expression (Fig. 3). Then on day 4 of culture the medium was replaced by one devoid of insulin and IGF-I but supplemented with FSH. In the absence of FSH, withdrawal of insulin and IGF-I decreased oestradiol accumulation and P450arom mRNA content to low or undetectable levels \( (P<0.02; \text{ Fig. 3}) \). When FSH was
added to the culture medium, oestradiol concentrations were re-established to amounts not different from controls (P > 0.05), but P450arom mRNA remained undetectable. Although there was a tendency to have higher concentrations of oestradiol with 1 ng FSH/ml than with the higher doses, this difference was not significant (P > 0.05). Oestrone (not shown) and oestradiol were correlated (r = 0.8, P < 0.001, n = 15), but neither were correlated with P450arom mRNA levels (P > 0.05).

In contrast to the effects on P450arom mRNA content, the withdrawal of insulin and IGF-I did not decrease P450scc mRNA levels (P > 0.05; Fig. 4). The addition of FSH increased progesterone accumulation and P450scc mRNA levels (P < 0.01). Progesterone concentrations and P450scc mRNA abundance were correlated (r = 0.6, P < 0.05, n = 15).

The above studies showed a lack of correlation between P450arom mRNA levels and oestradiol accumulation. To determine whether steroid accumulation is influenced by extant enzyme or short-term changes in gene expression, cells were recovered at several time-points after insulin withdrawal and FSH replacement. P450arom mRNA levels increased transiently at 6 h after medium change, and decreased (P < 0.001) to barely detectable levels by 24 h (Fig. 5). Oestradiol accumulation in the medium increased (P < 0.05) during the first 6 h, but did not change thereafter (Fig. 5).

Discussion

During the early growth of follicles in cattle, there is a period of time during which the future dominant follicle and largest subordinate follicle are similar in size and/or growth rates (Mihm et al. 2000, Ginther et al. 2001a). It has been suggested that these two follicles respond differently to the same FSH stimulus, and that this difference allows the future dominant but not the largest subordinate follicle to continue growing when FSH concentrations are low (Mihm et al. 2000). The earliest predictors of the future dominant follicle appear to be higher oestradiol and free IGF-I concentrations (reviewed by Fortune et al. 2001); however, it is not known if these two factors are causally related. In this study, we attempted to mimic in vitro the effect of declining FSH or insulin-like stimulus. Our results clearly show that a reduction of insulin and IGF-I caused a major decline in P450arom mRNA levels in oestrogenic bovine granulosa cells. These data thus support a crucial link between changes in IGF and oestradiol secretion: a decrease in insulin-like bioavailability can specifically inhibit aromatase expression, and is a potential cause rather than effect of regression of the future subordinate follicle.

An important observation in the present work is the apparent essential role of insulin for P450arom expression. When insulin or both insulin and IGF-I concentrations...
were reduced to low physiological levels, P450arom mRNA abundance was reduced to very low levels or was undetectable by Northern blotting. Equally novel is the finding that FSH did not prevent this loss of P450arom gene expression, even at supraphysiological doses. This is in contrast with data obtained with rat (Fitzpatrick & Richards 1991) and human (Steinkampf et al. 1987) granulosa cells, in which P450arom mRNA levels could be induced by FSH in the absence of insulin, IGF-I and serum. This may reflect differences between these species, or it may reflect differences in the culture conditions, including the FSH concentrations used. This requirement for insulin is also specific for P450arom and not a general metabolic effect on the cells, as FSH was quite capable of stimulating P450scc mRNA levels and progesterone accumulation in the absence of insulin. This latter observation also suggests that the inability of FSH to stimulate P450arom after withdrawal of insulin and IGF-I is not caused by a loss of FSH receptors.

Withdrawal of only IGF-I did not affect P450arom mRNA levels or steroid secretion, but this is likely because of the concentrations of insulin remaining in the medium. When insulin was reduced to low physiological levels (approximately 1 ng/ml; Richards et al. 1989) after withdrawal of insulin alone, IGF-I maintained P450arom mRNA levels at approximately 50% of control values. When IGF-I was also withdrawn and reduced to levels comparable to free IGF-I concentrations in subordinate bovine follicles (Beg et al. 2001), P450arom mRNA levels declined to less than 5% of control values. Thus, in the presence of low physiological concentrations of insulin, a reduction in IGF-I concentrations had a negative impact on P450arom mRNA levels. This suggests that the insulin concentrations used here may be too high, such that IGF-I becomes important only when insulin concentrations are below 10 ng/ml. In our laboratory, this dose of insulin resulted in the greatest sensitivity of granulosa cells to FSH (Bhatia & Price 2001). A confounding factor is the bioavailability of IGF-I. Non-luteinizing granulosa cells express IGFBP-2 mRNA and are positive for IGFBP-2 and -4 (of thecal origin) protein (Armstrong et al. 1998). These binding proteins associate with the extracellular matrix and sequester IGF-I, possibly providing an extracellular store of IGF-I. Follicles also contain IGFBP proteases, which may be regulated by hormones including FSH (Rivera et al. 2001). We do not know if the IGF-I administered in the present study was sequestered and thus unavailable to the cells, or sequestered and released slowly during the period of IGF-I withdrawal.

Following the reduction of insulin (or insulin and IGF-I), FSH apparently maintained oestradiol secretion despite dramatic reductions in P450arom mRNA levels. A time-course analysis indicated that the loss of P450arom mRNA starts to occur 12 h after the medium change, thus permitting oestradiol production from extant enzyme. Thus this model is suited for measuring mRNA accumulation, but caution should be exercised when interpreting steroid accumulation in media. Bearing this caveat in mind, it is nevertheless interesting to note that oestradiol accumulation after insulin withdrawal is significantly reduced if FSH is not replaced, suggesting that FSH may have some transient effect on P450arom mRNA that was not observed after 48 h of culture.

It is also interesting to note that after the withdrawal of FSH, insulin and IGF-I, cells appeared to contain less 28S rRNA (Fig. 3). These blots were also hybridized with a gliceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, and this mRNA was also reduced in the insulin- and FSH-devoid cells (data not shown). It is possible that under these conditions the cells were becoming atretic. The addition of FSH prevented this apparent decline in 28S and GAPDH, although it was unable to restore P450arom expression. This is consistent with reports showing that FSH decreases atresia in ruminant granulosa cells (Adams et al. 1993, Jablonka-Shari et al. 1996).

It should be noted that the control of aromatase in rats and cattle differs in important ways. Oestradiol secretion from cultured granulosa cells is stimulated by high concentrations of LH and FSH in rats but inhibited in cattle

![Figure 5](image-url)
(Berndtson et al. 1995), and P450arom gene expression and oestradiol secretion occurs in luteinized granulosa cells and corpora lutea in rats (Hickey et al. 1990, Gonzalez-Robayna et al. 1999) but not in cattle (Hinshelwood et al. 1993, Gutiérrez et al. 1997). Thus, acute control of aromatase activity and P450arom gene expression may be important in cattle, but less so in rodents. This ‘tight’ control of aromatase activity would contribute to the divergence in oestradiol concentrations between future dominant and largest subordinate follicles (Mihm et al. 2000), and to the rapid decrease observed following the LH surge in cattle (Voss & Fortune 1993).

P450sc mRNA levels and progesterone secretion were regulated by changes in FSH but not insulin or IGF-I. The essential role of FSH has been shown by a number of studies (Berndtson et al. 1995, Silva & Price 2000). The lack of major effects of insulin and IGF-I withdrawal is not in general agreement with the literature, as several studies showed a stimulatory effect of insulin on progesterone secretion from granulosa cells in vitro (reviewed by Spicer & Echternkamp 1995). However, in most of those studies, the cells spontaneously luteinized in culture. In the non-luteinizing cell model used herein, insulin did not stimulate P450sc mRNA expression or progesterone secretion (Silva & Price 2000).

The main hypothesis for the mechanism of follicle deviation in cattle proposes that the future dominant follicle becomes more sensitive to FSH, and can grow in a low-FSH environment. Recent evidence suggests that IGF-I plays an important role in this process. According to one model, there is an increase in IGFBP protease activity in the future dominant follicle which decreases IGFBP concentrations, whereas IGFBP concentrations remain high in subordinate follicles (Fortune et al. 2001). Decreased IGFBP activity raises free IGF-I concentrations, which in turn maintain follicle cell steroidogenesis and growth despite decreased FSH concentrations, and the follicle becomes dominant. In an alternative model, subordinate follicles produce greater amounts of IGFBP than the future dominant follicle (Austin et al. 2001) and thus are exposed to a decrease in free IGF-I concentration (Beg et al. 2001). In this latter model, decreased IGF bioavailability would be a cause rather than an effect of decreasing oestradiol synthesis and follicle growth. The present data support this hypothesis, as a decrease in IGF-I and overall insulin-like bioavailability decreased P450arom expression and aromatase activity.

Acknowledgements

We thank Cathérine Brière, Lynda Jourdain and Mélanie Hamel for assistance with cell cultures and assays, Drs A K Goff, G Cooke and A Bélanger for steroid antibodies, Dr M R Waterman for the bovine P450sc mRNA, and Dr A F Parlow and the NIDDK National Hormone and Peptide Program for providing bovine FSH. This work was supported by NSERC Canada, and J M S was supported by CONACYT, Mexico.

References


Hickey GJ, Krasnow JS, Beattie WG & Richards JS 1990 Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3′,5′-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5′ genomic DNA. *Molecular Endocrinology* **4** 3–12.


Received in final form 20 May 2002
Accepted 29 May 2002