Hormonal regulation of cytochrome P450 aromatase mRNA stability in non-luteinizing bovine granulosa cells *in vitro*

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Abstract

In the present study, we determined the potential for post-transcriptional regulation of cytochrome P450 aromatase (Cyp19), cytochrome P450 side-chain cleavage (Cyp11a) and 17 β -hydroxysteroid dehydrogenase I (Hsd17b1) mRNA. Bovine granulosa cells were cultured in non-luteinizing conditions that permit long-term oestradiol secretion. Half-lives of mRNA were measured by Northern and/or reverse transcriptase (RT)-PCR after inhibition of gene transcription. In FSH-stimulated cells, the Cyp11a and Hsd17b1 mRNAs had half-lives greater than 12 h. The half-life of

Cyp19 mRNA was significantly shorter at 3 h. The addition of the translation inhibitor cycloheximide to FSH-stimulated cells significantly increased Cyp19 mRNA half-life to approximately 12 h. Stimulation of cells with insulin resulted in Cyp19 mRNA half-life that was double ($P < 0.05$) that in FSH-stimulated cells. We conclude that bovine Cyp19 mRNA is very labile under physiological conditions, and that Cyp19 expression is under hormonal control at a post-transcriptional level.

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Introduction

A key endocrine marker of differentiating ovarian granulosa cells is the ability to synthesize oestradiol (reviewed by Drummond & Findlay 1999, Rosenfeld *et al.* 2001). Oestrogen is necessary for the induction of luteinizing hormone (LH) receptors on granulosa cells (Knecht *et al.* 1985) and, as a consequence, aromatase knock-out (ArKO) mice do not ovulate (Fisher *et al.* 1998). Further studies with ArKO mice have shown that oestradiol also plays a role in the control of the size of the primordial follicle pool and oocyte diameter (Britt *et al.* 2004). In ruminants and humans, granulosa cells convert theca-derived androgens to oestrogens with the enzyme cytochrome P450 aromatase (Cyp19) (Simpson & Davis 2001), and may convert androstenedione to testosterone and/or oestrone to oestradiol with 17 β -hydroxysteroid dehydrogenase I (Hsd17b1) (Luu-The 2001). There are marked increases in oestradiol content (Badinga *et al.* 1992, Mihm *et al.* 2000) and in Cyp19 and Hsd17b1 mRNA abundance (Xu *et al.* 1995, Bao *et al.* 1997, Sahmi *et al.* 2004) as bovine follicles grow from small to large antral stages.

The primary stimulator of granulosa Cyp19 expression is follicle-stimulating hormone (FSH) (Steinkampf *et al.* 1987, Fitzpatrick & Richards 1991, Silva & Price 2000), which differentially regulates the expression of the genes encoding Cyp19 and cytochrome P450 cholesterol side-chain cleavage (Cyp11a) enzymes in both bovine and human granulosa cells

(Yong *et al.* 1994, Silva & Price 2000). It is also clear that insulin and/or insulin-like growth factor-I (IGF-I) play important roles in stimulating aromatase activity and Cyp19 mRNA levels in bovine and human granulosa cells *in vitro* (Willis *et al.* 1996, Gutiérrez *et al.* 1997, Silva & Price 2002, Spicer *et al.* 2002). Furthermore, hyperinsulinaemia increases follicular oestradiol secretion in cattle and humans (la Marca *et al.* 2002, Butler *et al.* 2004).

The abundance of cellular proteins such as steroidogenic enzymes is largely a reflection of mRNA levels, and these are controlled by the rate of gene transcription. However, regulation of mRNA can also occur at a post-transcriptional level, most notably through changed stability of short-lived mRNA of genes, such as those encoding the oestrogen receptor and the LH receptor (Saceda *et al.* 1998, Hirakawa *et al.* 1999). The apparent half-life of Cyp19 mRNA is between 4 and 7 h in rat (Fitzpatrick *et al.* 1997) and rabbit (Hanoux *et al.* 2003) granulosa cells, whereas in human granulosa cancer (Mu *et al.* 2001) and choriocarcinoma cells (Wang & Chen 1994) the half-life is 12 h. This discrepancy requires attention, as it may be a result of differences between polyovulatory (rats and rabbits) and monovulatory (human) species, or between cancerous and normal tissues.

The objectives of the present study were to determine the half-life of mRNA encoding the steroidogenic enzymes Cyp19, Hsd17b1 and Cyp11a in bovine granulosa cells. As we found that Cyp19 mRNA is relatively short-lived, we tested

the hypothesis that the stability of this mRNA is under hormonal control. To do so, we cultured bovine granulosa cells under conditions that maintain FSH-responsive oestradiol secretion (Gutiérrez *et al.* 1997, Silva & Price 2000) and in which luteinization does not occur (Sahmi *et al.* 2004). We also measured the effects on Cyp19 mRNA stability of other agents that are known to increase steady-state Cyp19 mRNA abundance in rats, namely blocking translation with cycloheximide (CHX) (Fitzpatrick *et al.* 1997) and blocking the p42/p44 mitogen-activated protein kinase (MAPK) pathway (Moore *et al.* 2001).

Materials and Methods

Cell culture

The cell culture system was based on that described previously (Gutiérrez *et al.* 1997) and used in our laboratory (Silva & Price 2000). All materials were obtained from Invitrogen Life Technologies unless otherwise stated.

Briefly, bovine ovaries were collected at a local abattoir and transported to the laboratory at 35 °C in PBS containing penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). In the laboratory, the ovaries were rinsed in ethanol, then in PBS (with antibiotics). Follicles were dissected free of surrounding tissue, and small follicles (2–5 mm diameter) were bisected in α -minimal essential medium (α MEM) at 37 °C. Granulosa cells were recovered by passing the follicle walls repeatedly through a 1 ml disposable pipette. Cells were washed three times in α MEM containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), and then resuspended in culture medium. Cell viability was estimated at 30–40% by Trypan Blue exclusion.

Cells were cultured in 24-well tissue culture plates (Falcon; Becton Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 10^6 viable cells/well in 1 ml α -MEM with L-glutamine containing sodium bicarbonate (10 mM), Hepes (20 mM), protease-free BSA (0.1%; Sigma), sodium selenite (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (10^{-7} M; Sigma), human recombinant IGF-I (10 ng/ml), insulin (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, 95% air for 6 days, with 700 µl medium being replaced every 2 days.

mRNA stability assay

Stability of mRNA was measured by Northern hybridization or reverse transcriptase (RT)-PCR after the addition of transcriptional inhibitors actinomycin D (ActD; 5 µg/ml; Sigma) or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB, 75 µg/ml; Sigma). These doses were chosen based on previous studies examining steroidogenic protein mRNAs (Genissel *et al.* 2001, Wickenheisser *et al.* 2005). Cells were cultured with FSH (AFP5332B; NHPP, Torrance, CA, USA; 1 ng/ml), insulin (100 ng/ml, without FSH) or

dibutyryl-cAMP (cAMP; 300 µM), and transcription inhibitors were added on day 6. Cells were recovered at the time points given in Results by the removal of medium and addition of 150 µl Trizol/well. Steady-state levels of mRNA were measured in the time-0 cultures. To determine the effects of CHX, cells were cultured with or without FSH and CHX (20 µg/ml) was added for 4 h before addition of DRB. To measure the effect of the specific MAPK inhibitor PD-98059, cells were cultured with or without FSH and PD-98059 (50 µM; Sigma) was added for 48 h before the addition of DRB. For logistical reasons, cultures in the absence of FSH (with or without CHX or PD-98059) were performed in different experiments to those containing FSH (or insulin and cAMP), but FSH-stimulated controls were included in all experiments. Cultures were terminated at the time points given in Results by the removal of medium and addition of 150 µl Trizol/well. The plates were stored at –80 °C until RNA extraction.

RNA extraction and Northern hybridization

Total RNA was extracted by the standard Trizol procedure. The relative abundance of mRNA was determined in some experiments by Northern hybridization. Electrophoresis of 15 µg RNA was performed through a 1% denaturing formaldehyde-agarose gel followed by overnight capillary transfer onto a nylon membrane (Hybond-N; Amersham). Membranes were UV cross-linked in a commercial UV chamber (Bio-Rad) and incubated for 2 h in prehybridization solution containing 10% dextran sulphate, 5-strength saline-sodium phosphate-EDTE buffer (SSPE), 5-strength Denhardt's solution, 0.5% SDS and herring sperm DNA (200 mg/ml).

Bovine Cyp19 (Soumano *et al.* 1996) and Cyp11a cDNA (Dr M R Waterman, Vanderbilt University School of Medicine, Nashville, TN, USA; John *et al.* 1984) probes were labelled with [α -³²P]dCTP by random primer extension using a kit from Boehringer Mannheim, and purified by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega). 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 then stripped and rehybridised to a labelled human 28S ribosomal (Mrps28) cDNA probe (Gonzalez *et al.* 1985) for the standardization of RNA loading. The labelled membranes were either exposed to Kodak X-Omat film at –70 °C in the presence of an intensifying screen and autoradiograms were scanned with a densitometer after 1–14 days exposure, or were exposed to a Kodak phosphor screen for 3 days and digitalized with a phosphorimager (Molecular Dynamics Storm 840; Amersham). Bands were quantified with NIH Image (autoradiograms) or ImageQuant software (Amersham).

RT-PCR

Separate reverse transcription reactions were performed for each target gene with 1 µg total RNA and OmniScript RT.

Reverse transcriptions were performed with oligo-d(T) primer at 50 °C for Hsd17b1, and with oligo-d(T) and gene-specific primer for Cyp11a (5'-AGGGACACTGGTGTGGAACATC-3') (Lenz *et al.* 2004) and Cyp19 (5'-GACTCTCATGAATTCTCCATACATCT-3') (Fürbass *et al.* 1997) at 42 °C. For PCR, the primers were 5'-CTGAAGCAACAGGAGTCCTAAATGTACA-3' (sense) and 5'-GGACTAGTAATGAGGGGCCCAATTCACAGA-3' (antisense) for Cyp19 (Fürbass *et al.* 1997), 5'-AGAGAATCCACTTTCGCACATC-3' (sense) and 5'-AGGGACACTGGTGTGGAA-CATC-3' (antisense) for Cyp11a (Lenz *et al.* 2004), and 5'-TCCGCCACCTCCTCAGGGTTCT-3' (sense) and 5'-ATGGCCCGCACCGTGGTGCTCA-3' (antisense) for Hsd17b1 (Sahmi *et al.* 2004). The housekeeping gene used in PCR was histone H2a (H2a; primers given in Robert *et al.* 2002). Cycling conditions for Cyp19 and Cyp11a were denaturing at 94 °C for 3 min, followed by 27 cycles of 94 °C for 45 s, 60 °C for 30 s and 72 °C for 1 min. Cycling conditions for Hsd17b1 were denaturing at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min. Cycling conditions for H2a were denaturing at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min. Preliminary experiments were performed to verify that PCR product intensity increased with amount of RNA in the reverse transcription reaction, and that PCR intensity increased with cycle number (Sahmi *et al.* 2004). Amplification was performed within the linear range for each primer pair, and PCR products were visualised on agarose gels after staining with ethidium bromide and quantified with NIH Image software.

Aromatase activity assay

Stability of aromatase activity was measured by stimulating cells with FSH (1 ng/ml) or insulin (100 ng/ml) for 6 days, and replacing the culture medium on day 6 with medium containing CHX (20 µg/ml). Samples were then collected at 4, 8, 12, 26 and 52 h after treatment. Control samples received normal medium without CHX and were collected at 4 and 52 h. Aromatase activity was measured by the tritiated water assay as validated in this cell culture system (Bhatia & Price 2001). Briefly, 2–3 pmol [1β - ^3H]androstenedione was added to each well 6 h before each sample collection time. At each time point, medium samples were collected and frozen before assay, and total cell protein harvested by incubating cells with 200 µl, 1 M NaOH for 4 h. The extract was neutralized with 200 µl, 1 M HCl, and cell protein quantified with the Bradford protein assay (Bio-Rad). The medium samples were extracted twice with 2× volume chloroform, and the amount of tritiated water in the aqueous phase was determined by liquid scintillation counting. The data were corrected for cell number (total protein) and expressed relative to the 4-h control.

Statistical analysis

The density of Northern hybridization signals was corrected for loading efficiency using hybridization to Mrps28 rRNA, and PCR data are expressed as the ratio between target gene

and H2a mRNA. Data were expressed relative to control (without inhibitor) samples. Half-lives of mRNA and enzyme activity were determined from semilog plots of mRNA/enzyme activity and time. Values for percentage of mRNA remaining after 12 h (analysis of Cyp11a mRNA) were arcsin-transformed before analysis. ANOVA was used to test effects of treatments on mean mRNA or aromatase activity levels, and on estimates of half-life. Culture replicate was included as a random variable in the *F*-test for the effect of experimental replicate. Differences between groups were identified with the Tukey-Kramer HSD test. Analyses were performed with JMP software (SAS Institute, Cary, NC, USA). The data are presented as the mean $\pm$ S.E.M. of three to four independent replicate cultures.

Results

In the first experiment, we measured steroidogenic enzyme mRNA decay over 24 h in FSH-stimulated cells following inhibition of transcription with DRB or ActD. The mean half-life of Cyp19 mRNA was 3.1 ± 0.5 and 2.6 ± 0.2 h for DRB (Fig. 1) and ActD respectively, as measured by Northern blot. There was no difference between these two inhibitors. The half-life of Cyp11a mRNA was approximately 14 h (Fig. 1) and again there was no difference between DRB and ActD treatments. The decay profile for Hsd17b1 mRNA was measured by PCR, as expression levels are low in cultured

Figure 1 Decay profile of Cyp11a and Cyp19 mRNA in non-luteinized bovine granulosa cells following treatment with the transcription inhibitor DRB. Target gene mRNA levels were measured by Northern blot, calculated relative to Mrps28 rRNA, and are expressed as a percentage of control cultures (no DRB). Data are least-squares means $\pm$ S.E.M. obtained from three replicate experiments. The insert shows a representative Northern blot.

granulosa cells, and the half-life following DRB inhibition of transcription was longer than 12 h (Fig. 2).

Measuring Cyp19 mRNA half-life by Northern analysis in primary cultures of bovine granulosa cells requires a large number of cells, so we measured mRNA half-life in the remaining experiments by semiquantitative RT-PCR. Analysis by RT-PCR provided the same estimate of Cyp19 decay as Northern blot (2.9 ± 0.1 vs 3.1 ± 0.5 h, respectively), indicating that PCR provided accurate estimates of stability. Measurement of Cyp11a mRNA by PCR also gave a decay profile similar to that measured by Northern blot (data not shown).

As Cyp19 mRNA is relatively short-lived, we assessed the potential for endocrine regulation of this mRNA. Cells were cultured with or without FSH, insulin or cAMP, and with the addition of CHX or PD-98059 to FSH-stimulated or unstimulated cells, and transcription was inhibited on day 6 of culture by the addition of DRB. We first analysed the effects of these treatments on Cyp11a mRNA stability as a control for potential nonspecific or toxic effects on the cells. Steady-state Cyp11a mRNA levels were not significantly affected by culture conditions (not shown). As the half-life of Cyp11a measured by PCR was longer than 12 h, we assessed the impact of cell stimulators and drugs on the relative amount of mRNA remaining at 12 h after addition of DRB. Stability was not affected by the addition of FSH, insulin or cAMP, or treatment with PD-98059 in the presence or absence of FSH (Fig. 3). Treatment of cells with CHX alone had a tendency to increase stability of Cyp11a mRNA relative to treatment with FSH ($P=0.06$; Fig. 3).

In the absence of FSH (irrespective of the presence of CHX or PD-98059), Cyp19 mRNA levels were undetectable and

could not be analysed further. Among the FSH-, insulin- and cAMP-treated groups, there were significant ($P<0.05$) effects of time, treatment and a time by treatment interaction on Cyp19 mRNA levels. Steady-state mRNA levels (measured at time-0, immediately before adding DRB) were significantly higher in FSH+PD-98059-treated cells relative to the other treatments (Fig. 4A). Stimulation of cells with insulin or cAMP resulted in greater Cyp19 mRNA stability compared with cells cultured with FSH or with FSH+PD-98059 ($P<0.05$, Fig. 4B), and CHX increased Cyp19 mRNA half-life to approximately 12 h ($P<0.01$, Fig. 4B).

Finally, we measured the stability of aromatase activity to determine if Cyp19 is regulated at the protein/enzyme activity level. Cells were stimulated with FSH or insulin, and aromatase activity was measured following the inhibition of *de novo* protein synthesis by CHX. There were significant ($P<0.05$) effects of time and treatment on enzyme activity, and a time by treatment interaction (Fig. 5). The treatment difference was caused by the higher basal aromatase activity under insulin stimulation compared with FSH stimulation, and not to altered activity half-life.

Discussion

The results of this study provide evidence for two important conclusions: the mRNA encoding Cyp19 is significantly more labile than those encoding the other granulosa steroidogenic enzymes, Cyp11a and Hsd17b1; and Cyp19 mRNA stability is under hormonal regulation.

Little is known about the post-transcriptional control of steroidogenic enzyme expression/activity. The mRNA encoding Cyp11a is considered to be fairly stable as no significant decline was observed over 6 h in JEG-3 cells (Brentano & Miller 1992), and the present data show that this mRNA is quite stable in primary culture of bovine granulosa cells (half-life approximately 14 h). This is somewhat longer than the 6–8 h half-life reported for mouse MA-10 Leydig tumour cells (Hum *et al.* 1993). The present data also indicate that Hsd17b1 mRNA is also stable; we are not aware of any other reports on the stability of this mRNA.

In contrast to Cyp11a and Hsd17b1 mRNA, the half-life of Cyp19 mRNA is quite short at approximately 3 h under FSH stimulation. These data are similar to the half-life of 4 h reported for rabbit (Hanoux *et al.* 2003) granulosa cells, although shorter than the 7 h half-life reported for rat granulosa cells (Fitzpatrick *et al.* 1997) and nonstimulated Leydig cells (Genissel *et al.* 2001). These estimates are in contrast to the 13–16 h half-life reported for human granulosa cancer (Mu *et al.* 2001) and placental choriocarcinoma cells (Wang & Chen 1994), although these long half-lives may reflect altered Cyp19 stability in cancerous cells rather than species-specific differences. As transcription inhibitors may have indirect effects also, we used two compounds that block transcription by different mechanisms (Sobell 1985,

Figure 2 Decay profile of Hsd17b1 mRNA in nonluteinized bovine granulosa cells following treatment with the transcription inhibitor DRB. Target gene mRNA levels were measured by semiquantitative RT-PCR, calculated relative to H2a mRNA, and are expressed as a percentage of control cultures (no DRB). Data are least-squares means $\pm$ S.E.M. obtained from three replicate experiments. The insert shows a representative PCR gel.

Figure 3 Stability of Cyp11a in stimulated and non-stimulated bovine granulosa cells *in vitro*. In one experiment, cells were cultured under basal conditions (B), or with the MAPK inhibitor PD-98059 (PD), with CHX (CHX), or with FSH. In another experiment, cells were stimulated with FSH, with dibutyl- α -cAMP (cAMP), with a high dose of insulin (100 ng/ml; INS), or with FSH plus PD-98059 (FSH + PD) or FSH plus CHX (FSH + CHX). The vertical line separates these two experiments. RT-PCR was used to estimate relative amount of mRNA at the time of addition of DRB and at 12 h post-DRB (relative to H2a mRNA as an internal control), from which the proportion of mRNA remaining at 12 h was calculated. Data are least-squares means $\pm$ S.E.M. obtained from three independent replicate cultures for each experiment.

Yamaguchi *et al.* 1998). Both DRB and ActD resulted in similar estimates of mRNA half-life. This, plus the consistent half-life values obtained in several culture experiments by Northern or by RT-PCR methods, suggests that these data reflect authentic mRNA degradation rates.

The stability of some mRNAs with short half-lives is regulated by hormonal or other stimuli (reviewed by Guhaniyogi & Brewer 2001). Rat Leydig cells express Cyp19 under basal nonstimulated conditions, and the addition of LH plus androgen significantly stabilised Cyp19 mRNA (Genissel *et al.* 2001). Bovine granulosa cells do not express Cyp19 mRNA under basal conditions (Silva & Price 2000) and therefore we could not assess stability under these conditions. Stimulation of granulosa cells with FSH or high concentrations of insulin (without FSH) induced Cyp19 mRNA expression as expected (Silva & Price 2000), and insulin increased mRNA stability compared with stimulation with FSH. Although no other data are available for ovarian steroidogenic enzyme mRNA stability, insulin has been shown to stabilize mRNA encoding IGF-I, vascular endothelial growth factor and angiotensin II receptor in other cell types (Nickenig *et al.* 1998, Bermont *et al.* 2001, Goya *et al.* 2001). The role of insulin in granulosa cell function is of practical/clinical interest as hypoinsulinaemia is associated with anovulation during the postpartum period and with undernutrition in cattle (Armstrong *et al.* 2003, Lucy 2003). In anovulatory women with polycystic ovary syndrome, insulin (*in vitro*) or hyperinsulinaemia (*in vivo*) stimulates granulosa cell oestradiol output (Willis *et al.* 1996, 1998, Franks *et al.* 2000). These effects of insulin may thus be

exerted in part at a post-transcriptional level involving regulation of Cyp19 mRNA stability.

Stimulation of cells with cAMP also resulted in a greater stability of Cyp19 mRNA compared with FSH stimulation. The effects of cAMP on mRNA stability is diverse depending on cell type, and has been shown to stabilize mRNA encoding renin in kidney cells (Morris *et al.* 2004) and vasopressin in the rat hypothalamus (Kuwahara *et al.* 2003). In rat and human granulosa cells, cAMP was reported to have no effect on the stability of mRNA encoding FSH receptor, Cyp11a and StAR (Hum *et al.* 1993, Kiriakidou *et al.* 1996, Tano *et al.* 1997), and had no effect on Cyp11a in the present study. Recently, it was shown that forskolin, an adenylyl cyclase stimulator, prolonged cytochrome P450 17 α -hydroxylase mRNA stability in human theca cells (Wickenheisser *et al.* 2005), which is consistent with the effects of cAMP observed on Cyp19 mRNA stability in the present study. The difference in mRNA stability in FSH- and cAMP-stimulated cells is interesting, as cAMP is the classical intracellular mediator of FSH action, including activation of protein kinase A and MAPK (Gonzalez-Robayna *et al.* 2000, Cottom *et al.* 2003). FSH may also act independently of cAMP, as FSH and cAMP had different effects on p42 MAPK activation in porcine granulosa cells (Cameron *et al.* 1996) and activation of MAPK through the growth factor-like type-3 FSH receptor occurred independently of cAMP (Babu *et al.* 2000). However, it is unlikely that the p42/44 MAPK pathway is involved in regulation of Cyp19 mRNA half-life under FSH stimulation as inhibition of this pathway with PD-98059 did not alter the half-life of the mRNA.

Inhibition of new protein synthesis with CHX resulted in considerable stabilization of Cyp19 mRNA, as previously

Figure 4 Hormonal regulation of Cyp19 mRNA stability. (A) Mean $\pm$ S.E.M. steady-state levels of Cyp19 mRNA in bovine non-luteinized granulosa cells cultured without FSH (C), or cultured with FSH (FSH), FSH plus the MAPK inhibitor PD-98059 (FSH + PD), FSH and CHX (FSH + CHX), dibutyl-cAMP (cAMP) or with insulin (INS). Data were obtained by RT-PCR from samples recovered at the time of DRB addition (time-0 in B), and expressed relative to H2a mRNA. Cyp19 mRNA was not detectable in cells cultured without FSH (nd), so these data were not analysed further. Asterisk indicates mean value different from all others stimulated groups ($P < 0.001$). (B) mRNA decay profiles of Cyp19 mRNA following the addition of the transcription inhibitor DRB. The treatment groups are as described in (A). Half-lives were calculated from semilog plots and mean $\pm$ S.E.M. half-life estimates ($T_{1/2}$; h) are shown for each treatment. Mean half-life estimates with different superscripts are different ($P < 0.05$).

observed in rat granulosa cells (Fitzpatrick *et al.* 1997). There was also a moderate stabilization of Cyp11a mRNA levels, suggesting that the action of CHX is not specific to Cyp19, and implies a general inhibition of RNase activity. Although CHX prolonged Cyp19 mRNA stability in the present study, it did not increase the steady-state mRNA levels as was reported in rat cells (Fitzpatrick *et al.* 1997). This difference is likely due to differences in the time of exposure of cells to CHX; in the former study, cells were exposed to CHX for 7 h before mRNA

stability measurement, whereas we exposed cells to CHX for only 4 h, which may not have provided sufficient time for significant accumulation of mRNA. It is also possible that the rate of Cyp19 transcription is lower in cultured cells from small bovine follicles compared with cells from preovulatory rat follicles, which would make detection of short-term changes in steady-state mRNA levels more difficult.

For the short half-life of Cyp19 to be physiologically relevant, it must be reflected by rapid changes in oestradiol

Figure 5 Stability of aromatase activity in FSH- (○) or insulin-stimulated (●) bovine granulosa cells. Aromatase was induced with FSH (1 ng/ml) or with insulin (100 ng/ml), and on day 6 of culture medium was replaced with medium containing the translation inhibitor CHX (20 µg/ml). Aromatase activity was estimated with the tritiated water assay at the time points shown (see Materials and Methods). Control cultures (with FSH but without CHX) were measured at 4 and at 52 h (■). Data were corrected for total cell protein and then expressed relative to the 4 h control cultures. Data are means $\pm$ S.E.M. of three replicate experiments.

synthesis/secretion. We tested this by adding the translation inhibitor CHX to FSH- or insulin-stimulated cells. In both cases, aromatase activity was reduced to 50% of control levels by 7–8 h, which implies that the stability of Cyp19 enzyme activity in primary bovine granulosa cells is shorter than that of the Cyp19 protein (12 h) in JEG-3 human choriocarcinoma cells (Harada *et al.* 1999). Comparison of protein and activity data should be made with caution, as confounding elements such as cofactors may alter enzyme activity but not protein concentrations.

In summary, the present data demonstrate hormone regulation of the stability of Cyp19 mRNA in bovine granulosa cells. Labile aromatase activity coupled with a short-lived mRNA indicates that constant stimulation is required to maintain mRNA levels and enzyme activity in bovine granulosa cells and perhaps other tissues. This may explain at least in part the sensitivity of small follicles to decreases in plasma FSH concentrations during the oestrous cycle (Fortune *et al.* 2001, Ginther *et al.* 2003). A fuller understanding of post-transcriptional regulation of Cyp19 mRNA may also lead to alternative methods for the manipulation of aromatase activity for fertility control and treatment of oestrogen-dependent tumours.

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