Levels of messenger RNA encoding ovarian receptors for FSH and LH in cattle during superovulation with equine chorionic gonadotrophin versus FSH

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Abstract

This study tested the hypothesis that luteal LH receptor (LHr) and follicular LHr and FSH receptor (FSHr) steady-state mRNA levels are greater during superovulation with equine chorionic gonadotrophin (eCG) compared with that with FSH. Heifers were stimulated with eCG ($n=10$) or FSH ($n=10$), and ovaries were recovered the day before and at 12 and 24 h after luteolysis was induced with prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$). Total RNA was purified from individual follicles and corpora lutea. Steady-state levels of LHr and FSHr mRNA were assessed by slot blot analysis employing homologous cDNA probes. There were no differences in luteal LHr between FSH- and eCG-stimulated animals before luteolysis, and hybridization signals were detected in only one of six animals by 12 h

after injection of PGF $_{2\alpha}$. After PGF $_{2\alpha}$ injection, steady-state levels of follicular LHr were 4-fold lower ($P<0.05$) and follicular FSHr mRNA levels were 2.4-fold lower ($P<0.05$) in eCG- compared with FSH-treated cattle. In eCG-treated animals, induction of luteolysis led to a significant increase in follicular LHr mRNA levels ($P<0.01$) and a significant decrease in follicular FSHr mRNA levels ($P<0.01$). There was no such effect of luteolysis in FSH-treated animals. We conclude that superovulation with eCG, compared with FSH, results in lower follicular levels of LHr and FSHr mRNA but does not affect luteal LHr mRNA levels.

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Introduction

In cattle, plasma concentrations of progesterone and oestradiol are higher during superovulation with equine chorionic gonadotrophin (eCG) than with follicle-stimulating hormone (FSH) preparations low in luteinizing hormone (LH) bioactivity (Armstrong *et al.* 1983, Driancourt & Fry 1992, Ben Jebara *et al.* 1994). In cattle, steroid biosynthesis is under the control of LH in luteal cells (Niswender & Nett 1994) and both LH and FSH in follicular cells (Gore-Langton & Armstrong 1994). These glycoprotein hormones act through specific receptors; once bound with ligand, the hormone–receptor complex activates the adenylate cyclase pathway (Marsh & LeMaire 1974, Davis 1994, Hillier *et al.* 1994, Laurent-Cadoret & Guillou 1995) leading to stimulation of the steroidogenic pathway. The first step in gonadotrophin-stimulated steroidogenesis is the transfer of cholesterol from the outer to the inner mitochondrial membrane, where it is converted to pregnenolone by cytochrome P450 cholesterol side-chain cleavage (P450 $_{sc}$) (reviewed by Miller 1988, Niswender & Nett 1994, Niswender *et al.* 1994). This transport may be mediated by several factors (reviewed by Hall & Almahbobi 1992, Jefcoate *et al.* 1992), of which

steroidogenic acute regulatory (StAR) protein is believed to be the rate-limiting step for steroidogenesis (Hartung *et al.* 1995, Stocco & Clark 1996, Stocco 1997). Pregnenolone is converted to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD) in corpora lutea (CL) and in follicles (reviewed by Labrie *et al.* 1992), or to androgens and subsequently to oestradiol in follicles (Fortune 1986, Miller 1988).

We have recently shown that ovarian hyperstimulation with eCG increases, compared with stimulation with FSH, levels of mRNA for follicular, but not luteal, StAR protein (Pescador *et al.* 1996, Soumano & Price 1997). This may be specific to theca cells, because StAR protein is weakly expressed in non-luteinized bovine granulosa cells (Soumano & Price 1997), and follicular cytochrome P450 17 α -hydroxylase (P450 $_{17\alpha}$) mRNA levels were increased in eCG- compared with FSH-stimulated cows (Soumano *et al.* 1996). The expression of StAR protein is acutely regulated by LH (Clark *et al.* 1995, Juengel *et al.* 1995), thus modifications of StAR protein mRNA levels may reflect changes in the LH signal to the cell, which may also involve LH receptor number and receptor synthesis.

It has been shown that gonadotrophin regulation of bovine (Houde *et al.* 1994) and rat ovarian FSH receptor

(FSHr) (LaPolt *et al.* 1992) and LH receptor (LHr) (LaPolt *et al.* 1990, 1991, Nakamura *et al.* 1990, Segaloff *et al.* 1990) content during follicular growth, ovulation and luteinization is associated with similar changes in their respective receptor message levels. For instance, induction of follicular growth in rats with eCG increased ovarian LHr and FSHr numbers and mRNA content (LaPolt *et al.* 1990, 1992), and FSH caused a dose-dependent increase in FSHr mRNA content in cultured rat granulosa cells (Tilly *et al.* 1992).

The aim of this study was to test the hypothesis that, during superovulation, the higher LH activity of eCG, compared with a commercial FSH preparation (Folltropin), stimulates an increase in steady-state levels of mRNA for LH receptors in luteal cells as well as mRNA for LH and FSH receptors in follicular cells.

Material and Methods

Animals and treatments

The study was conducted on twenty, 18-month-old crossbred Holstein–Friesian heifers as previously described (Soumano *et al.* 1996). Animals were housed indoors at the CRRA farm in St-Hyacinthe, Québec, Canada (lat 45° 30' N), and were maintained under standard husbandry conditions. Observation for oestrus was conducted daily to verify that the animals were displaying regular oestrous cycles before the beginning of the treatments. The animals were divided into two groups ($n=10$ /group) for super-ovulation with either eCG or FSH. The treatments began on day 9 of the cycle (day 0=day of oestrus). The eCG-treated animals received one i.m. injection of 2500 IU Folligon (Intervet Canada Inc., West Hill, ON, Canada). The FSH-treated group received, at 12-h intervals, eight i.m. injections of 2.25 mg Folltropin-V (Vetrepharm Inc., London, ON, Canada; equivalent to 50 mg NIH-FSH-P1). On the morning of day 12, animals received an i.m. injection of 500 µg prostaglandin $F_{2\alpha}$ (PGF_{2α}: Cloprostenol; Estrumate, Cooper Agropharm Inc., Ajax, ON, Canada) to induce luteal regression.

Plasma progesterone and oestradiol concentrations have been reported for these treatments in these animals in a previous superovulatory cycle (Soumano *et al.* 1996).

RNA extraction

Ovaries were removed from hyperstimulated heifers by colpotomy on day 11 or 12 (before PGF_{2α}, $n=4$ heifers/treatment), and at 12 h ($n=3$ heifers/treatment) and 24 h ($n=3$ heifers/treatment) after PGF_{2α} injection. CL and all follicles greater than 3 mm were removed within 30 min of ovariectomy, and were placed in denaturing solution (4 M guanidine isothiocyanate, 0.12 M β-mercaptoethanol and 20 mM sodium acetate, pH 6.0) and stored at −70 °C until total RNA was extracted.

The thawed tissues were homogenized in denaturing solution, and total RNA was isolated by centrifugation for 21 h at 174 000 g on a cushion of 5.7 M caesium chloride as described by Davis *et al.* (1986). The pellet was resuspended in 3 M sodium acetate, pH 5.2, precipitated twice with ethanol and quantified by spectrophotometry at 260 nm. Total RNA was analyzed by slot blot hybridization (6 µg) as previously described (Sambrook *et al.* 1989). One slot blot contained all CL samples whereas follicular samples were distributed across four blots. Membranes were then cross-linked (150 mJ) in a UV chamber (Bio Rad, Mississauga, ON, Canada) and incubated for 2 h in prehybridization solution, containing 10% dextran sulphate, 5 × saline–sodium phosphate–EDTA (SSPE: 0.75 M NaCl, 50 mM NaH₂PO₄·H₂O, 5 mM EDTA, pH 7.4), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin), 0.5% sodium dodecyl sulphate (SDS), and herring sperm DNA (200 mg/ml) (Sambrook *et al.* 1989). The CL samples were hybridized with labelled bovine LHr cDNA probe while blots containing follicular samples were sequentially hybridized with labelled bovine LHr and FSHr cDNA probes. Hybridization was performed overnight at 65 °C. After hybridization, membranes were washed in 2 × SSPE–0.1% SDS twice at room temperature, and twice at 65 °C. Membranes were then stripped and rehybridized to a human 28 S ribosomal RNA (rRNA) probe 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 (Collage Analysis; Fotodyne Inc., Hartland, WI, USA) after 1 to 5 days exposure.

cDNA probes

The bovine LHr cDNA probe was generated by reverse transcription and polymerase chain reaction (RT-PCR). The probe used corresponds to a 786 bp cDNA fragment of the amino terminal extracellular binding domain (from exon 11) up to the seventh transmembrane spanning segment (nucleic acids 1087 to 1899 of the genebank sequence deposited by Lussier *et al.* 1995). The bovine FSHr cDNA probe was a 1264 bp fragment generated by RT-PCR and representing a portion of the extracellular binding domain to the 3' stop codon (Houde *et al.* 1994). The 28 S rRNA probe was a 1.4 kb portion from an internal region of the human gene (Gonzalez *et al.* 1985). All probes were labelled with [α -³²P]dCTP, to a specific activity of 1.5–3.0 × 10⁹ d.p.m./µg, by random primer extension using a kit (Boehringer Mannheim, Laval, PQ, Canada). Labelled probes were separated from unincorporated [³²P]dCTP by centrifugation through a minicolumn using Wizard PCR Preps DNA purification system (Promega, Montreal, PQ, Canada).

Statistical analysis

The density of hybridization signals was corrected for loading efficiency using hybridization to 28 S rRNA, and for follicular samples, these corrected data were normalized relative to a follicular quality control sample (pooled RNA from bovine follicles) that was included in all four slot blots (coefficient of variation of the quality control sample between blots was <20%). Follicles were classified by size into small (3–6 mm), medium (6–9 mm) and large (>9 mm) and the mean steady-state levels of each RNA within follicle size group were calculated (in dimensionless units) for each animal. As the data did not follow a normal distribution by the Shapiro–Wilk test, they were transformed to common logarithms, and analysis of variance (ANOVA) was used to test effects of time relative to PGF_{2α} injection, gonadotrophin treatment and time by treatment interaction. Additionally, for follicular samples, the effect of follicle size was assessed. Comparisons between individual groups were made with the Tukey–Kramer HSD test. Analyses were performed with JMP software (SAS Institute, Cary, NC, USA). The data are presented as arithmetic mean ($\pm$ S.E.M.) mRNA levels relative to 28 S rRNA.

Results

Luteal mRNA levels

There was a significant main effect of time but not of treatment on steady-state luteal LHr mRNA levels ($P<0.05$). Before luteolysis, LHr mRNA levels were not significantly different between FSH (11.0 ± 3.8 relative units) and eCG (623.6 ± 614.0 relative units) treated animals ($P>0.05$); the apparently large value for the eCG group was caused by one animal showing an extremely high level of LHr mRNA. Levels of mRNA for LHr decreased significantly ($P<0.001$) after PGF_{2α} injection, such that at 24 h after PGF_{2α} injection only one eCG-treated animal showed a detectable hybridization signal (1.4 relative units), and no animals showed detectable signals at 24 h after PGF_{2α}.

Follicular mRNA levels

There were no effects of follicle size or interactions between follicle size and time or treatment on steady-state levels of mRNA for LHr or FSHr in follicles (illustrated for 24 h after PGF_{2α} in Fig. 1). Consequently, all further analyses were performed with the mean value of all follicles (irrespective of size).

There was a significant main effect of treatment ($P<0.05$) on levels of mRNA for LHr, these being 3- to 5-fold lower overall in eCG- compared with FSH-stimulated animals (Fig. 2). However, an effect of treatment at each time point did not reach significance, owing

Figure 1 Steady-state mRNA levels (relative to 28 S rRNA) of LHr and FSHr in large (L; >9 mm), medium (M; 6–9 mm) and small (S; 3–6 mm) follicles from cattle undergoing ovarian hyperstimulation with FSH or eCG. These data, which serve to illustrate the similarity across follicle size groups within treatment, are for 24 h after luteolysis was induced by injection with prostaglandin F_{2α}. Each bar represents the mean of three cows except the mean for small follicles from the eCG-treated group, in which two cows contributed follicles.

in part to the large variation among the FSH-treated animals. For eCG-stimulated cows, LHr mRNA levels increased with time, and were 2.4-fold higher at 24 h after, compared with before, PGF_{2α} injection ($P<0.01$; Fig. 2). No such effects of time were observed between

Figure 2 Effect of treatment and of time relative to prostaglandin $F_{2\alpha}$ injection on steady-state levels of LHr mRNA (relative to 28 S rRNA) in follicles from cattle undergoing superovulation with FSH (○) and eCG (●). Within treatments, different letters denote significant differences ($P < 0.05$). The main effect of treatment was significant ($P < 0.05$). $n = 4$ at 0 h, and $n = 3$ at 12 and at 24 h.

Figure 3 Effect of treatment and of time relative to prostaglandin $F_{2\alpha}$ injection on steady-state levels of FSHr mRNA (relative to 28 S rRNA) in follicles from cattle undergoing superovulation with FSH (○) and eCG (●). Different letters denote significant differences ($P < 0.05$) across times and treatments. $n = 4$ at 0 h, and $n = 3$ at 12 and at 24 h.

follicles collected before and after $PGF_{2\alpha}$ injection in FSH-treated heifers ($P < 0.05$; Fig. 2).

There was a significant main effect of treatment and a time-by-treatment interaction for levels of mRNA encoding FSHr ($P < 0.05$). There was a significant effect of time relative to $PGF_{2\alpha}$ injection on levels of mRNA for FSHr in eCG-treated heifers, such that levels after the induction of luteolysis decreased to 58% of the levels observed before luteolysis ($P < 0.01$; Fig. 3). There was no significant effect of time on FSHr mRNA levels in FSH-stimulated cattle. At 12 and 24 h after luteolysis, the relative levels of FSHr mRNA in follicles from FSH-treated animals were two to three times higher than those in follicles from eCG-treated animals ($P < 0.05$; Fig. 3).

Discussion

The aim of this study was to test the hypothesis that, during superovulation, the higher LH activity of eCG, compared with a commercial FSH preparation (Folltropin), stimulates an increase in steady-state levels of mRNA for LH receptors in luteal cells as well as mRNA for LH and FSH receptors in follicular cells. The main observations from this study are that: (1) there were no significant differences between eCG and FSH treatments on the levels of LHr mRNA in CL and (2) after $PGF_{2\alpha}$ injection, superovulation with eCG, compared with FSH, resulted in a lower level of follicular LH and FSH receptor mRNA.

The lack of a treatment difference in LHr mRNA in CL is perplexing. The obvious endocrine effect of eCG-induced superovulation, compared with saline or FSH, is increased plasma progesterone concentrations, most noticeable before luteolysis (Ben Jebara *et al.* 1994, Soumano & Price 1997). However, this is not accompanied by changes in levels of mRNA encoding luteal LHr (present study), StAR protein or $P450_{scc}$ (Pescador *et al.* 1996), or 3β -HSD (K Soumano & C A Price, unpublished observations). Collectively, these data suggest that increased plasma concentrations of progesterone are a result of an LH-dependent mechanism downstream of mRNA (e.g. protein stability and/or enzyme activity; Belfiore *et al.* 1994). An alternative explanation is that increased progesterone concentrations are of follicular origin. Some evidence from the literature supports this idea. Treatment with eCG, compared with FSH, led to greater intrafollicular progesterone secretion in goats *in vivo* (Kumar *et al.* 1992) and in short-term culture in sheep (Driancourt & Fry 1992). However, in the present study, there were no treatment effects on follicular LHr (or FSHr) mRNA levels before luteolysis, and we have shown that follicular StAR protein, $P450_{scc}$ and 3β -HSD mRNA levels are not different between these two treatments before luteolysis (Soumano *et al.* 1996, Soumano & Price 1997). So again, the increase in progesterone concentrations at this time is most likely effected downstream of steady-state mRNA levels.

Plasma oestradiol concentrations following luteolysis are significantly higher in animals stimulated with eCG compared with those stimulated with FSH (Ben Jebara *et al.* 1994, Soumano *et al.* 1996). The present data now show that follicular gonadotrophin receptor mRNAs are lower in eCG-stimulated compared with FSH-stimulated animals after luteolysis, coincident with increases in follicular StAR protein and P450_{17 α} mRNA levels (Soumano *et al.* 1996, Soumano & Price 1997). As StAR protein is acutely regulated by LH (Stocco 1997), the increased level of StAR protein mRNA suggests increased stimulation by eCG even though receptor mRNA levels are reduced.

The lower steady-state FSHr and LHr mRNA levels during eCG compared with FSH treatment suggest either down-regulation of these receptors during eCG treatment (Hoffman *et al.* 1991), or up-regulation during FSH treatment. Low doses of FSH and eCG have been shown to increase FSH and LH binding sites and FSHr and LHr mRNA levels in rats and mice (LaPolt *et al.* 1990, 1992, Segaloff *et al.* 1990, Tilly *et al.* 1992, Wang & Greenwald 1993a,b), although low and high levels of FSH decreased FSHr mRNA levels in bovine granulosa cells *in vitro* (Houde *et al.* 1994). Down-regulation of FSHr and LHr mRNA is usually only observed with high (ovulatory) levels of eCG or hCG (LaPolt *et al.* 1990, 1992, Segaloff *et al.* 1990). Thus it is possible that the eCG dose regimen used to superovulate cattle is high enough to cause down-regulation of LHr and FSHr mRNA levels. This may be supported by the increased incidence of luteinization observed in cattle undergoing superovulation with eCG (Boland *et al.* 1991).

Interestingly, eCG had an opposing effect on LHr and FSHr mRNA levels; FSHr mRNA decreased with time after the induction of luteolysis, whereas LHr mRNA levels increased with time. This may be related to the relative FSH:LH activities of eCG in cattle; although eCG binds to both receptors, the predominant activity is that of FSH (Murphy & Martinuk 1991), so it may bind to FSHr to a greater extent than to LHr. In this case, down-regulation of FSHr with a concomitant up-regulation of LHr may occur.

The induction of luteolysis caused a marked decrease in luteal LHr mRNA levels. This decrease is coincident with that observed for StAR protein mRNA (Pescador *et al.* 1996), and supports an acute response of StAR protein to the loss of LH receptors, and to the uncoupling of the receptor from the cAMP second messenger cascade reported during this time in bovine CL (Pate & Condon 1984). The induction of luteolysis also resulted in an increase in follicular LHr mRNA and a decrease in FSHr mRNA levels in eCG-stimulated cattle. This phenomenon can probably be interpreted as reflection of the normal physiological process of luteinization. A higher incidence of follicle luteinization has been observed in eCG-stimulated than in Folltropin-stimulated cattle (Boland *et al.* 1991). During the luteinization process,

bovine follicular cells lose their ability to express FSHr (Houde *et al.* 1994, Rajapaksha *et al.* 1996) while developing LHr needed for LH-dependent progesterone synthesis (Niswender & Nett 1994).

In summary, this study has demonstrated that, during superovulation of cattle, stimulation with eCG compared with FSH results in lower steady-state mRNA levels for FSHr and LHr in follicles. Treatment with eCG resulted in an increase in LHr mRNA and a decrease in FSHr mRNA levels following the induction of luteolysis. There were no differences between these superovulatory treatments in their effects on luteal LHr mRNA levels.

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