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The progesterone receptor antagonist, onapristone has differential effects on the timing and control of the luteolytic mechanism depending on timing of administration in sheep

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ABSTRACT

Cyclic ewes were treated with control vehicle or progesterone receptor antagonist (onapristone; 100 mg i.m. twice daily) during either early (day 3–5) or late (day 12–14) luteal phase and plasma samples collected for hormone analysis and to determine endogenous and oxytocin induced $PGF_{2\alpha}$ release. On day 14 and 17, ewes were euthanised and reproductive tracts collected for ovarian morphology and endometrium for oxytoxin and steroid hormone receptor analysis. Early treatment increased LH, but not progesterone or oestradiol, while late treatment elevated all three hormones. Early treatment delayed the up-regulation of endometrial oxytocin receptors and responsiveness to oxytocin challenge, delaying luteolysis. Late treatment advanced development of oxytocin receptors and responsiveness to oxytocin though not timing of luteolysis. Patterns of hormone receptor mRNA were differentially disrupted by treatments. Results provide mechanistic insight into hormonal control of the oestrous cycle and identify the ability of the luteolytic mechanism to dissociate from functional luteolysis.

1. Introduction

The action of progesterone is necessary in stimulating the endometrial infrastructure to support early pregnancy. In ruminants, it is, however, also an integral component in the preparation of the luteolytic mechanism by stimulating the accumulation of phospholipid stores and increasing phospholipase A2 and prostaglandin G/H synthase 2 (PTGS2) expression in the uterine epithelia. This is required for the production of luteolytic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

It has long been hypothesised that the timing of luteolysis is determined by the length of exposure to progesterone during the luteal phase (Geisert et al., 1992; Spencer and Bazer, 1995; McCracken et al., 1999). This is supported by studies in cyclic ewes and cows that have clearly demonstrated that administration of exogenous progesterone during the early luteal phase, to advance the post ovulatory progesterone rise, results in an earlier onset of luteolysis and shortening of the cycle (Woody et al., 1967; Ottobre et al., 1980; Garrett et al., 1988) through alterations in uterine steroid receptor populations (Okumu et al., 2010). However, delaying the post-ovulatory progesterone rise is more technically demanding. Work in ovariectomized cows treated with progesterone and oestradiol to recreate typical cyclic changes results in the development of responsiveness to oxytocin, in terms of $PGF_{2\alpha}$ release, from around day 16 of the simulated cycle (Lamming and Mann, 1995). As expected, advancing the simulated progesterone rise advanced the time of onset of responsiveness to oxytocin (Mann et al., 1998). However, delaying the administration of progesterone by 3 days to delay the simulated progesterone rise in this model system did not result in any delay the onset of responsiveness to oxytocin (Mann et al., 1998). This suggests that a simple timing mechanism set by the time of the progesterone rise is not the only controlling mechanism and that other associated mechanisms remain to be elucidated. Furthermore, in cyclic cows, a delayed endogenous rise in progesterone is not followed by any delay in the timing of luteolysis and, in fact, the luteal phase is shortened (Lamming and Darwash, 1995). The key component in the initiation of the luteolytic mechanism is the up-regulation of oxytocin receptors (OXTR) in the endometrium (Spencer and Bazer, 1995; Mann et al., 1999). It is widely accepted that oestrogens can up regulate OXTR mRNA and protein in a number of species and, in the ewe, exogenous administration of oestradiol increases endometrial OXTR concentrations (Hixon and Flint, 1987; Spencer et al., 1996). It is also well established that, following the initiation of luteolysis, the resultant preovulatory rise in oestradiol during the follicular phase is associated with a further dramatic rise in OXTR. However, *ex vivo* studies have remained equivocal as to whether prior

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up-regulation of endometrial oestrogen receptor 1 (ESR1) initiates OXTR up-regulation during the initiation of the luteolytic mechanism in ruminants (Wathes and Hamon, 1993; Spencer and Bazer, 1995; Spencer et al., 1996; Robinson et al., 1999). It is recognised in the ewe that OXTR is a default receptor which rises in the absence of inhibition, for example following ovariectomy (Vallet et al., 1990). Thus, it is possible that the rise associated with the initiation of luteolysis is merely the result of the withdrawal of progesterone mediated inhibition rather than the stimulatory action of oestradiol.

Onapristone ((ZK-98299; 11β-(4-dimethylaminophenyl)-17α-hydroxypropyl-17β-(3-hydropropinyl)-13α-methyl-4,9-gonadiene-3-one) is one of a large number of selective progesterone receptor (PGR) modulators (SPRMs) that have been developed as potential therapeutic clinical agents. It shares a similar structure to mifepristone, the original and most widely used of the SPRM's and is a pure PGR antagonist with no agonist activity (Chabbert-Buffet et al., 2005). Onapristone affects both PGR-A and PGR-B isoforms of the progesterone receptor. The aim of this study was to identify the molecular and endocrine mechanisms through which blocking the action of progesterone disrupts the development of the luteolytic mechanism and functional luteolysis in the intact cycling ewe.

2. Materials and methods

2.1. Experimental animals and treatments

All studies were undertaken during December and January in mature cyclic Dorset ewes (53.2 ± 1.3 kg) housed in pens under natural daylight and were fed a maintenance diet of hay and cereal pellets. All procedures were carried out under the Animals (Scientific Procedures) Act 1986. Oestrus was initially synchronised by administering 2 injections of 100 μg cloprostenol i.m. (0.4 ml Estrumate; Schering-Plough Animal Health, Welwyn Garden City, UK) 12 days apart. On the day of the 2nd injection, a jugular vein of each ewe was cannulated under local anesthesia and these were maintained for the duration of the experiment and used for the collection of all blood samples.

Treated ewes were administered the progesterone receptor antagonist, onapristone (ZK-98299, Schering AG, Berlin, Germany), made up at a concentration of 50 mg/ml in 25% benzyl benzoate: 75% castor oil. Treatment was administered as a series of 2 ml i.m. injections according to the specific treatment group. Control ewes received equivalent 2 ml i.m injections of the vehicle. The dose used was based on that of Walker et al. (1997) who established 2 mg/kg as an effective dose of onapristone for use in the ewe.

2.2. Study 1: effect of onapristone administration during the early luteal phase

In this study, treated ewes ($n = 4$) were administered onapristone (100 mg) at 8 am and 8 pm on days 3 and 4 and at 8 am on day 5 of the cycle; control ewes ($n = 4$) received equivalent injections of vehicle.

Blood samples were collected daily from the day of oestrus (d 0) until slaughter on day 17. On days 14, 15 and 16, additional blood samples were collected at hourly intervals for 12 h in both control and treated ewes, for measurement of plasma concentrations of 13, 14 dihydro-15-keto $PGF_{2\alpha}$ (PGFM), the principle metabolite of $PGF_{2\alpha}$. Prior to slaughter on day 17, ewes were injected with a single i.v. bolus of oxytocin (5 i.u. in 0.5 ml saline; Hoechst UK Ltd., Milton Keynes, UK) to monitor endometrial responsiveness to oxytocin. The oxytocin was administered via the jugular cannula, which was then flushed with a further 5 ml saline. Blood samples were collected at 20 min intervals for 1 h before and at 10 min intervals for 1 h after administration of oxytocin.

On day 17, ewes were euthanised with an overdose of sodium pentabarbitone (Euthatal; RMH Animal Health, Dagenham, Essex, UK) and reproductive tracts collected to record gross ovarian morphology and collect uterine endometrium for oxytocin binding analysis and steroid hormone receptor mRNA analysis. For oxytocin binding analysis, strips of endometrium were cut from sites across the uterine horn and pooled area samples stored at −80 °C for subsequent analysis. For hormone receptor localisation, uterine cross sections were taken from the middle portion of the uterine horn. Previously, we have shown that there are no differences in OXTR concentrations in the bovine endometrium from the upper, middle or lower regions of the uterus (Mann and Lamming, 1994). The uterine cross sections were wrapped in foil and snap-frozen in isopentane cooled in liquid nitrogen before storage at −80 °C until processed for receptor localisation.

2.3. Study 2: effect of onapristone administration during the late luteal phase

In this study, treated ewes ($n = 4$) were administered onapristone (100 mg) at 8 am and 8 pm on days 12 and 13 and at 8 am on day 14 of the cycle: control ewes ($n = 4$) received equivalent injections of vehicle. As in Study 1, blood samples were collected for hormone analysis (LH, E2 and P4) and to determine endogenous and oxytocin induced $PGF_{2\alpha}$ release. Ewes were once again euthanised on day 17 and reproductive tracts collected to record ovarian gross morphology and uterine endometrial hormone receptor analysis.

2.4. Study 3: effect of early and late treatment with onapristone on the luteolytic development on day 14

To further elucidate the underlying mechanisms, an additional study was undertaken in which ewes (control, early and late) were slaughtered on day 14. In this study, ewes were treated with onapristone (100 mg) from day 3–5 (Early; $n = 4$) or day 12–14 (Late; $n = 4$) according to the protocols in Studies 1 and 2 or acted as controls ($n = 4$) treated with vehicle on days 12–14. On day 14, all ewes underwent oxytocin challenge and were then euthanised and tissues collected as in Studies 1 and 2.

2.5. Sample analysis

LH was measured in plasma by radioimmunoassay (Mann and Lamming, 2000) using NIDDK-anti-oLH-1, iodinated NIDDK-oLH-I-2 and a bovine LH standard (bLH AFP11743B) supplied by Dr. A.F. Parlow (Pituitary Hormones and Antisera Center, Harbor UCLA Medical Center, Torrance, CA 90509, USA). Intra- and inter-assay coefficients of variation were 9.6% and 11.3% respectively and the sensitivity of the assay was 0.2 ng/ml. Progesterone was measured in plasma after extraction with diethyl ether by radioimmunoassay (Hunter et al., 1986). Intra- and inter-assay coefficients of variation were 6.3% and 8.6% respectively and the sensitivity of the assay was 0.2 ng/ml. Oestradiol was measured in plasma following diethyl ether extraction using a radioimmunoassay kit (Serono Diagnostics Ltd., Woking, Surrey, UK) modified to increase sensitivity (Mann et al., 1995) and validated for use in the sheep by Beard et al.,(1994). The sensitivity of the assay was 0.5 pg/ml and the intra and inter-assay coefficients of variation were 7.1% and 9.6% respectively. Plasma PGFM concentrations were determined by the radioimmunoassay of Kaker et al. (1984) following extraction of duplicate aliquots of 500 μl plasma with acidified diethyl ether. Intra- and inter-assay coefficients of variation were 12.3% and