The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus

L A Okumu, N Forde, A G Fahey, E Fitzpatrick, J F Roche, M A Crowe and P Lonergan

School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Correspondence should be addressed to P Lonergan; Email: pat.lonergan@ucd.ie

Abstract

To investigate the effects of pregnancy or post-ovulatory progesterone (P_4) supplementation on the expression of oestrogen and P_4 receptors (ESRs and PGRs) in the bovine uterus, heifers ($n=263$) were randomly assigned to the following treatments: i) cyclic, normal P_4 ; ii) cyclic, high P_4 ; iii) pregnant, normal P_4 ; and iv) pregnant, high P_4 on days 5, 7, 13 and 16 of pregnancy/oestrous cycle. Elevated P_4 was achieved through P_4 -releasing intravaginal device insertion on day 3 after oestrus, resulting in increased concentrations from day 3.5 to 8 ($P<0.05$) in the high groups than in the normal groups. Irrespective of treatment, *PGR* and *ESR1* mRNA expressions were highest on days 5 and 7 and decreased on day 13 ($P<0.05$), while *ESR2* mRNA expression increased on day 7 ($P<0.05$) and similar levels were maintained within the normal P_4 groups subsequently. Expression in the high P_4 groups decreased on day 13 ($P<0.05$). *PGR*-AB and *PGR*-B protein expressions were high in the luminal and superficial glands on days 5 and 7, but by day 13, expression had declined to very low or undetectable levels and high P_4 concentration tended to decrease or decreased significantly ($P<0.05$) the expression in these regions on days 5 and 7. *ESR1* protein expression was high, with no treatment effect. *ESR2* protein was also highly expressed, with no clear effect of treatment. In conclusion, early post-ovulatory P_4 supplementation advances the disappearance of *PGR* protein from the luminal epithelium on days 5 and 7, and decreases *ESR2* mRNA expression during the mid-luteal phase, but has no effect on *PGR* or *ESR1* mRNA expression.

Reproduction (2010) **140** 143–153

Introduction

Reproductive tract and behavioural changes during the oestrous cycle of cattle are to a large extent governed by follicular oestradiol (E_2) and luteal progesterone (P_4) concentrations (Kimmins & MacLaren 2001). Conceptus growth and development require the action of P_4 on the uterus to regulate endometrial function including conceptus–maternal interactions, pregnancy recognition and uterine receptivity for implantation. The genomic effects of these two steroids on the endometrium are mediated primarily by the binding to their nuclear receptors: oestrogen receptor (ESR) and P_4 receptor (PGR) respectively, which are located within the target cells in the uterus (Ing & Tornesi 1997). Both belong to a superfamily of nuclear receptors that include not only steroid receptors but also vitamin D, thyroid hormone and orphan receptors (Carson-Jurica *et al.* 1990, Tsai & O'Malley 1994). The basic structure of these receptors consists of a C-terminal or ligand-binding domain, a highly conserved DNA-binding domain and an N-terminal domain of variable length (Stormshak & Bishop 2008).

Oestrogen enhances the expression of its own receptors as well as those for P_4 (Spencer & Bazer 1995, Wathes *et al.* 1996, Ing & Tornesi 1997), and functions at the cellular level to increase the rate of mitosis, tissue oedema and number of immune cells such as neutrophils and macrophages in the uterus (De & Wood 1990, Kachache *et al.* 1991, Tibbetts *et al.* 1999). P_4 is essential for ensuring a favourable uterine environment and maintaining pregnancy (Spencer *et al.* 2008, Forde *et al.* 2009). Contrary to the actions of oestrogen, P_4 is inhibitory to the expression of ESRs, and negatively regulates the expression of its own receptors during the mid-luteal phase of the oestrous cycle and during early pregnancy (Spencer & Bazer 1995, Spencer *et al.* 1995).

E_2 mediates its functions through the α -form (ESR1) and β -form (ESR2) of its receptors (D'Haeseleer *et al.* 2006). These forms differ in the N-terminal transactivation domain and in the C-terminal ligand-binding domain (Kuiper *et al.* 1997). ESR1 protein is expressed in the pituitary gland, ovary, uterus, testis, epididymis and adrenal gland (Kuiper *et al.* 1997), while ESR2

protein is mainly expressed in the ovary and the prostate (Kuiper *et al.* 1996, 1997, Enmark *et al.* 1997). P₄ mediates its genomic functions mainly through two kinds of nuclear receptors: PGR isoform A (PGR-A), which is N-terminally truncated (missing 164 amino acids), and PGR isoform B (PGR-B). Both isoforms are encoded by the same gene, but are regulated by different promoters (Mote *et al.* 2006). In different species, the two PGRs may function either individually as homodimers, e.g. in rodents (Mulac-Jericevic & Conneely 2004, Mote *et al.* 2006), or as a heterodimer, as is found in humans (Arnett-Mansfield *et al.* 2004). PGR-A plays a major role in mediating the actions of P₄ in the uterus and ovary, while PGR-B is more important in mammary gland development (Mulac-Jericevic *et al.* 2000, 2003).

P₄ is a key hormone that regulates conceptus development in ruminants. Elevated concentrations of P₄ in the immediate post-ovulatory period have been shown to significantly alter the pattern of endometrial gene expression by advancing the temporal changes that occur normally as well as by advancing elongation of the conceptus (Garrett *et al.* 1988, Satterfield *et al.* 2006, Carter *et al.* 2008). This effect has been shown to be a consequence of priming of the endometrium, presumably resulting in alterations of the histotroph composition, rather than to be a direct effect on the conceptus (Clemente *et al.* 2009). In support of this hypothesis, administration of P₄ early in the oestrous cycle can advance uterine receptivity for the transfer of older asynchronous embryos (Geisert *et al.* 1991).

Despite the importance of P₄ for the establishment and maintenance of pregnancy in mammals, paradoxically, endometrial epithelia cease expressing PGR prior to implantation in all mammals studied (Bazer *et al.* 2009). For example, loss of PGR in ovine endometrial luminal, superficial and glandular epithelia occurs by days 11 and 13 of the oestrous cycle and pregnancy respectively, but stromal cells and myometrial cells express PGR throughout gestation. In this study, it is hypothesised that elevated P₄ in the early luteal phase will lead to a more rapid decline in PGR in the uterine luminal epithelium (LE) of cyclic and pregnant heifers. This would lead to an alteration in the timing of expression of P₄-regulated genes in the endometrium, which has been shown to advance conceptus elongation (Garrett *et al.* 1988, Satterfield *et al.* 2006, Carter *et al.* 2008). Therefore, the objectives of this study were to determine i) the mRNA expression pattern and protein localisation of the oestrogen and nuclear PGRs in the uterus during different stages of the oestrous cycle; ii) how the expression and localisation patterns are affected by the presence of an embryo/conceptus and iii) if elevated P₄, which has been shown to advance conceptus development (Carter *et al.* 2008), alters the expression and/or localisation of these genes and proteins.

Results

PGR mRNA expression and protein localisation

In general, the expression pattern of both steroid receptors was not greatly affected by the presence or absence of a conceptus (i.e. pregnant versus cyclic endometrium), but it was strongly influenced by the day of the oestrous cycle/early pregnancy. In general, irrespective of the treatment group, expression of PGR mRNA was highest on day 5 and declined on subsequent days, with a significant decrease between day 7 and 13 (Fig. 1; $P < 0.05$). On a given day, the only differences between treatments were observed on day 13, when PGR mRNA abundance was lower in the pregnant animals than in the cyclic animals, irrespective of P₄ status ($P < 0.05$).

Generally, the intensity of staining was greater for the PGR-B isoform than for the PGR-A isoform. However, both exhibited the same pattern of expression, where the protein intensity declined as the days of pregnancy/oestrous cycle progressed (Figs 2 and 3). In the LE and superficial glands (SGs), protein levels of both PGR isoforms declined to very low or undetectable levels between day 7 and 13 in both pregnant and cyclic heifers, remaining so until day 16. This decline was significant only in the cyclic and pregnant groups with normal P₄ ($P < 0.05$).

Elevated P₄ promoted the early downregulation of the PGR on days 5 and 7 compared with normal P₄ concentrations. In the presence of elevated P₄, irrespective of pregnancy status, the disappearance of both protein isoforms from the LE and SGs was more rapid on days 5 and 7 than in the control animals; for example, animals with high P₄ on day 5 had approximately the same protein abundance as those with normal P₄ on day 7 (Figs 2 and 3).

Figure 1 Temporal and spatial expressions of PGR mRNA relative to ACTB (normaliser gene) in bovine endometrium. Expression values are given for cyclic, normal P₄ (CN; open bars); cyclic, high P₄ (CH; closed bars); pregnant, normal P₄ (PN; dotted open bars); and pregnant, high P₄ (PH; hatched closed bars) heifers on days 5, 7, 13 and 16 of the oestrous cycle/early pregnancy. Significant differences ($P < 0.05$) in PGR expression within specific treatments between days are denoted by A–D, while significant differences ($P < 0.05$) in PGR expression within each day amongst the treatments are denoted by x and y.

Figure 2 Representative images depicting the localisation of PGR-AB protein (A) in the luminal epithelium (LE), superficial glands (SGs) and stroma (STR) of bovine endometrium on days 5, 7, 13 and 16 in pregnant (P) heifers with normal (PN) or high (PH) P_4 . Bar = 100 μ m. Graphical representation of the intensity of PGR-AB protein isoform localised within the luminal epithelium (LE; B) or superficial glands (SGs; C) in uterine cross sections of cyclic and pregnant heifers with normal or high P_4 on days 5, 7, 13 and 16 of the oestrous cycle/early pregnancy. Significant differences ($P < 0.05$) in protein abundance are depicted with different superscripts between treatments within a specific day (x and y) and within treatment between days (A–C).

ESR1 and ESR2 mRNA expression and protein localisation

Expression of *ESR1* mRNA displayed a similar pattern to that of *PGR*, with it being high on days 5 and 7 and lower on day 13 ($P < 0.05$), and remaining low on day 16 (Fig. 4A). Expression of *ESR2* mRNA increased in all treatment groups from day 5 to 7 ($P < 0.05$). This level of expression was maintained to day 16 in animals with

normal P_4 . In contrast, elevated P_4 led to a decrease in expression on day 13 ($P < 0.05$, Fig. 5A).

ESR1 protein was detected in the LE, SGs, deep glands (DGs), myometrium (MYO) and stroma (STR), and was unaffected by day, pregnancy status or P_4 concentration ($P > 0.05$, Fig. 4B). The abundance of *ESR2* protein was also high in the uterus, with no clear pattern of difference in expression.

Figure 3 Representative images depicting the localisation of PGR-B protein isoform (A) in the luminal epithelium (LE), superficial glands (SGs) and stroma (STR) of bovine endometrium on days 5, 7, 13 and 16 in pregnant heifers with normal (PN) or high (PH) P₄. Bar = 100 µm. Graphical representation of the intensity of PGR-B protein isoform localised within the luminal epithelium (LE; B) or superficial glands (SGs; C) in uterine cross sections of cyclic and pregnant heifers with normal or high P₄ on days 5, 7, 13 and 16 of the oestrous cycle/early pregnancy. Significant differences ($P < 0.05$) in protein abundance are depicted with different superscripts between treatments within a specific day (x and y) and within treatment between days (A and B).

Discussion

P₄ and E₂ are important hormones in the regulation of the reproductive process in mammals. Not only are the concentrations of these hormones in peripheral blood important, but also their binding to specific nuclear receptors within target cells is critical for the biochemical action of P₄ at cellular level. In this study, for the first time, the mRNA expression and protein localisation patterns of the ESRs and the nuclear PGRs during the bovine oestrous cycle have been

characterised, and alterations in expression by the presence of a conceptus during early pregnancy and/or elevated P₄ concentrations on their expression were determined. In this study, it has been shown that the tissue distribution of steroid receptors is regulated during the oestrous cycle and early pregnancy in a cell-specific manner, and that elevated P₄ induces early down-regulation of PGR, which is a critical event for the expression of genes required for conceptus development, maternal recognition of pregnancy and

Figure 4 Temporal and spatial expressions of *ESR1* mRNA relative to *ACTB* (normaliser gene) in bovine endometrium (A). Expression values are given for cyclic, normal P₄ (CN; open bars); cyclic, high P₄ (CH; closed bars); pregnant, normal P₄ (PN; dotted open bars); and pregnant, high P₄ (PH; hatched closed bars) heifers on days 5, 7, 13 and 16 of the oestrous cycle/early pregnancy. Significant differences ($P < 0.05$) in *ESR1* expression within specific treatments between days are denoted by A–C, while significant differences ($P < 0.05$) in *ESR1* expression within each day amongst the treatments are denoted by x and y. Representative images (B) depicting the localisation of *ESR1* protein isoform in the luminal epithelium (LE), superficial glands (SGs) and stroma (STR) of bovine endometrium on days 5, 7, 13 and 16 in pregnant heifers with normal (PN) or high (PH) P₄. Bar = 100 μ m.

implantation. In the current study, although the whole endometrial homogenate used for quantitative real-time (QRT-PCR) could not demonstrate the cell-specific changes in mRNA expression, localisation of the receptor proteins through immunohistochemistry (IHC) analysis made this possible.

P₄ plays a key role in embryo/conceptus development and in the maintenance of pregnancy (McNeill *et al.* 2006, Satterfield *et al.* 2006). The *PGR* mRNA and protein expressions displayed similar temporal changes during the oestrous cycle/early pregnancy, and were consistent with other reports in cattle (Robinson *et al.* 2001) and sheep (Wathes & Hamon 1993, Spencer & Bazer 1995), with the highest expression being observed during the early luteal phase and lowest expression during the mid-late luteal phase (days 13 and 16). By day 16, *PGR* gene expression had begun increasing significantly in pregnant heifers. In this study, although *PGR*-AB was present at a much lower intensity than *PGR*-B, the pattern of expression for both proteins was similar, showing variation within specific cell types for specific days of oestrous cycle/early pregnancy. Highest expression of both *PGR*-AB and *PGR*-B proteins was

found in heifers with normal P₄ during the early luteal phase in agreement with previous findings in cattle (Kimmins & MacLaren 2001, Robinson *et al.* 2001, Martin *et al.* 2008) and sheep (Spencer & Bazer 1995); however, we have shown for the first time in cattle that high P₄ advances the downregulation of its own receptor in the LE and SGs, further supporting the hypothesis that high P₄ negatively regulates the expression of its own receptor. The intensity of both *PGR*-AB and *PGR*-B proteins localised within the DGs, MYO and STR remained detectable throughout, with varying levels of expression. Despite the fact that P₄ is critical for the establishment and maintenance of pregnancy, loss of the *PGR* from the endometrial epithelia is critical for the establishment of implantation (Bazer *et al.* 2009). In the current study, downregulation of *PGR* had occurred by day 13 consistent with previous findings (Spencer & Bazer 1995, Kimmins & MacLaren 2001), and coincides with the period of conceptus elongation, interferon τ secretion and maternal recognition of pregnancy (Spencer & Bazer 1996). The loss of *PGR* in uterine epithelia appears to be a prerequisite for maternal recognition of pregnancy and conceptus development

Figure 5 Temporal and spatial expressions of *ESR2* mRNA relative to *ACTB* (normaliser gene) in bovine endometrium (A). Expression values are given for cyclic, normal P₄ (CN; open bars); cyclic, high P₄ (CH; closed bars); pregnant, normal P₄ (PN; dotted open bars); and pregnant, high P₄ (PH; hatched closed bars) heifers on days 5, 7, 13 and 16 of the oestrous cycle/early pregnancy. Significant differences ($P < 0.05$) in *ESR2* expression within specific treatments between days are denoted by A–D, while significant differences ($P < 0.05$) in *ESR2* expression within each day amongst the treatments are denoted by x and y. Representative images (B) depicting the localisation of *ESR2* protein isoform in the luminal epithelium (LE), superficial glands (SGs) and stroma (STR) of bovine endometrium on days 5, 7, 13 and 16 in pregnant heifers with normal (PN) or high (PH) P₄. Bar = 100 μ m.

in early pregnancy (Spencer *et al.* 2008) as well as for epithelial cell proliferation and differentiated functions as directed by specific factors (progestagens) produced by PGR-positive stromal cells (Bazer *et al.* 2009). While P₄ supplementation beginning on day 3 had no significant effect on *PGR* mRNA expression, it had a profound effect on the expression of PGR protein on days 5 and 7. The fact that the level of expression of PGR-AB in the high P₄ group on day 5 was similar to the day 7 expression by the normal P₄ heifers demonstrates that high P₄ advanced the downregulation of PGR protein from mainly the LE. Low PGR expression in animals with elevated P₄ has been observed in sheep (Satterfield *et al.* 2006), but it had not been studied previously in cattle. This downregulation of PGRs by elevated P₄ during this critical window raises the questions of the mechanism of action by which elevated P₄ on days 3–7 subsequently is responsible for advanced conceptus elongation. High P₄ concentrations have been shown to advance conceptus elongation in cattle (Mann *et al.* 2006, Carter *et al.* 2008) and sheep (Satterfield *et al.* 2006). Similarly, transfer of embryos to a uterus previously exposed to elevated P₄

results in larger conceptuses on days 13 and 16 (Lonergan *et al.* 2007, Clemente *et al.* 2009). In addition, high P₄ levels have also been associated with advancement of P₄ actions in the uterus (McNeill *et al.* 2006, Forde *et al.* 2009). Drawing from these previously observed effects of high P₄ and the results from this study, we hypothesise that early priming of the uterus by P₄ through exogenous P₄ supplementation leads to an advanced downregulation of PGR from the LE and subsequently the SGs, allowing for early regulation of P₄-responsive genes in the uterus, that are geared towards advancing conceptus development and producing an optimum uterine environment for implantation. The P₄ profiles in the animals used in the current study had been fully characterised in a previous study (Carter *et al.* 2008), and were found to be significantly higher in the high P₄ groups than in the normal P₄ groups from day 3.5 until day 7, coincident with the time of decreased expression of PGR protein in the high P₄ groups. Thereafter, the P₄ concentration between the high and normal P₄ groups tended to merge (Carter *et al.* 2008). High levels of PGR protein are expressed during the early

luteal phase of the cycle, when P_4 concentrations although increasing are still low, and therefore unable to inhibit the stimulatory effects of E_2 on the expression of not only PGR but also ESR1. However, continuous exposure of the endometrium to P_4 for 8–10 days results in the downregulation of PGR from the endometrial LE and SGs (Spencer & Bazer 1995). In spite of this, high P_4 concentrations are still maintained until the late luteal phase when the luteolytic mechanism is activated in cyclic animals (Spencer & Bazer 1995). The luteal phase is said to be sustained by the action of progestagens on the PGR-negative cells (Spencer & Bazer 1995, Bazer *et al.* 2008). These progestagens, such as the P_4 -responsive fibroblast growth factors, are produced by uterine stromal cells, which still maintain PGR expression even after downregulation of PGR from LE and SGs has occurred (Spencer & Bazer 1995, Bazer *et al.* 2008).

E_2 is the other key steroid reproductive hormone, but its function relates more to stimulation of motility and secretory activity of the tract than to conceptus physiology (Spencer & Bazer 1995). The expression of ESR1 only differed between days 7 and 13, with a decrease in expression being maintained during the late luteal phase, which reflects the pattern described in previous studies in cattle and sheep (Wathes & Hamon 1993, Spencer & Bazer 1995, Robinson *et al.* 2001). This pattern of ESR1 expression mimics circulating concentrations of E_2 during the oestrous cycle (Sunderland *et al.* 1994). The E_2 concentrations are significantly elevated on days 3–6 from the first dominant follicle, but are not elevated from the second dominant follicle due to luteal phase P_4 concentrations suppressing LH pulse frequency during the late luteal phase of the oestrous cycle/early pregnancy. Although there was similar expression for ESR1 on days 5, 7 and 16 across all treatments, on day 13 in the current study, pregnant heifers had lower ESR1 expression than cyclic heifers. This is in contrast to earlier reports that found similar expression of the gene throughout the oestrous cycle and early pregnancy (Wathes & Hamon 1993, Spencer & Bazer 1995, Robinson *et al.* 2001). A previous inconsistency in the expression of ESR1 between the cyclic and pregnant groups on day 16 was suggested to be due to a difference in the length of the cycle between the animals used (Robinson *et al.* 2001). Another study (McNeill *et al.* 2006) found P_4 supplementation to increase ESR1 expression on day 6, and proposed it to be due to advancement of P_4 actions. This report contrasts with our current findings, in which P_4 supplementation had no significant effect on the expression of either PGR or ESR1 mRNA. From the current results, we suggest that the consequence of these advanced effects of high P_4 on ESR1 may at the level of translation lead to increased ESR1 protein due to a shortened cycle, perhaps pointing towards a faster development of the luteolytic mechanism as E_2 is partially luteolytic in late

dioestrus (Spencer & Bazer 1995). This is further supported by similar expression levels of ESR1 mRNA in the cyclic and pregnant heifers with high and normal P_4 treatments.

Throughout the period from day 5 to 16, the level of ESR2 in the current study was lower than that of ESR1. In fact, the lowest recorded level of ESR1 was still greater than the highest recorded level of ESR2. In rat uteri, ESR2 levels were also found to be much lower than ESR1 levels (Shughrue *et al.* 1998, Wang *et al.* 1999, Weihua *et al.* 2000), in spite of the uterus being a major oestrogen target tissue (Weihua *et al.* 2000). It has been suggested that ESR1 protein is more important than ESR2 protein for mediating oestrogen actions in the uterus (Pelletier *et al.* 2000). Although it has been reported previously in other species that both ESR subtypes have similar expression patterns for both mRNA and protein, differing only in the intensity of expression (Matsuzaki *et al.* 1999, Wang *et al.* 2000), the results obtained in this study show for the first time that in the bovine uterus, ESR1 and ESR2 mRNAs have different expression patterns during the oestrous cycle/early pregnancy. The actual role and mechanism of action of ESR2 within the reproductive tract as well as the role that both oestrogen and P_4 may play in its regulation are unknown (Wang *et al.* 2000). However, reduced fertility in ESR2 knockout female mice was associated with a likely loss of function of ESR2 gene (Kuiper *et al.* 1997, Wang *et al.* 2000). In the current study, ESR2 mRNA expression exhibited temporal regulation. Levels significantly increased on day 7 across all treatments. Previous studies in rats showed no association between the circulating hormone levels during the oestrous cycle and expression of either ESR2 mRNA or ESR2 protein in the uterus (Wang *et al.* 2000, Weihua *et al.* 2000). The current results indicate that in cattle, ESR2 may be responsive to the stimulatory effects of E_2 from a non-ovulatory follicle. Furthermore, results indicate that the normal physiological concentration of luteal P_4 has no modulatory effect on the expression of ESR2 in the bovine uterus, further emphasising its lesser role in the tissue as has been suggested previously (Shughrue *et al.* 1998, Wang *et al.* 1999, 2000). Although expression of ESR2 protein in the bovine uterus was higher than that found in other species, its expression pattern was similar to that of other species described previously and was unaffected by the stage of the cycle (Saji *et al.* 2000, Wang *et al.* 2000).

In conclusion, the current results characterise both ESR and PGR mRNA levels and protein within specific cell types in the uterus, and show that they are temporally regulated during the oestrous cycle and early pregnancy. For the first time, we have shown that high P_4 concentrations do not affect the expression of PGR or ESR1 mRNA, but did decrease ESR2 mRNA expression. Moreover, we have demonstrated for the first time that elevated P_4 from days 3–7 of the oestrous

cycle/early pregnancy advances the downregulation of the nuclear PGR in the LE, a key regulator of endometrial gene expression, which contributes, in part, to advanced conceptus development in cattle.

Materials and Methods

Animal model and tissue collection

Unless otherwise stated, all chemicals and reagents were sourced from Sigma. All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1897) and European Community Directive 86/609/EC, and sanctioned by the Animal Research Ethics Committee of University College Dublin. The experimental design was as described previously (Carter *et al.* 2008). In summary, the oestrous cycles of 263 heifers were synchronised using a controlled internal drug-releasing (CIDR, 1.94 g P₄) device (InterAg, Hamilton, New Zealand), which remained in the vagina for 8 days. Three days prior to CIDR device removal, all heifers received an injection of 2 ml of a prostaglandin F_{2α} analogue (Estrumate, Schering-Plough Animal Health, Hertfordshire, UK; equivalent to 0.5 mg cloprostenol) i.m. Of the 210 heifers that displayed standing oestrus within a narrow time window, 140 were artificially inseminated with semen to generate a pregnant group, while the remaining heifers were left as a non-inseminated cyclic control group. On day 3 of the oestrous cycle/early pregnancy, half of each group were randomly assigned to receive a P₄-releasing intravaginal device (1.55 g P₄; CEVA, Animal Health Ltd, Chesham, UK) to elevate circulating concentrations of P₄ (Carter *et al.* 2008). This resulted in four treatment groups: i) pregnant, high P₄ (PH); ii) pregnant, normal P₄ (PN); iii) cyclic, high P₄ (CH) and iv) cyclic, normal P₄ (CN). The heifers were then randomly assigned for slaughter on either day 5, 7, 13 or 16 of the oestrous cycle or early pregnancy. Within 30 min of slaughter, the reproductive tracts of all heifers were retrieved and flushed with 20 ml of PBS containing 10% FCS. In relation to the inseminated heifers, only tissues from those with an appropriately developed embryo/conceptus for the day of pregnancy were further processed. Thus, for each treatment, five animals were collected per time point (i.e. 4 treatments × 4 days × 5 animals = a total of 80 animals). Approximately one 25-mm whole cross section of the uterine horn ipsilateral to the corpus luteum was fixed for 24 h in 10% buffered

formalin for IHC analysis. Samples for IHC analysis were then processed by dehydration through a series of ascending concentrations of alcohol, cleared in xylene and finally impregnated with paraffin wax prior to sectioning for IHC analysis. Strips of endometrial tissue (~300 mg in weight) were removed from the mid-section of the ipsilateral horn, and were immersed in 1:5 w/v RNAlater and transported back to the laboratory on ice for QRT-PCR analysis. Samples for QRT-PCR were stored at 4 °C for 24 h, excess RNAlater was removed and placed into a new tube and stored at –80 °C prior to RNA extraction.

Quantitative real-time PCR

Changes in gene expression were analysed using QRT-PCR as described previously (Forde *et al.* 2009). Briefly, 100-mg samples of ipsilateral endometrium were homogenised and total RNA was extracted using Trizol reagent (Invitrogen) as per the manufacturer's instructions. On-column DNase digestion and RNA cleanup were performed using a Qiagen mini kit (Qiagen). Both quality and quantity of the RNA were determined using the Agilent bioanalyser (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesised from 5 µg of total RNA using Superscript III (Invitrogen) and random hexamers as per the manufacturer's instructions. All primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA) and manufactured by Eurofins MWG (Ebersberg, Germany). All reactions were performed using 50 ng of cDNA, 10 µl of SYBR Green Master Mix (Applied Biosystems) and primers at the optimised concentrations (Table 1). β-Actin (*ACTB*) was used as the normaliser gene, as determined by previous optimisation in this sample set (Forde *et al.* 2009). Final reaction volumes were made up to a total volume of 20 µl with RNase DNase-free H₂O. All QRT-PCRs were carried out in duplicate on the 7500 Fast RT-PCR System (Applied Biosystems). The cycling conditions were 50 °C for 2 min, 95 °C for 10 min, 40–95 °C cycles for 15 s each and 60 °C for 1 min. Inclusion of a dissociation curve in each QRT-PCR run ensured specificity of the amplicons.

Immunohistochemistry of *ESR1*, *ESR2*, *PGR-AB* and *PGR-B*

Protein localisation and intensity were determined using IHC analysis, using a previously described procedure (Van den Broeck *et al.* 2002), which we optimised to suit the current

Table 1 Accession numbers and symbols of genes used to generate primer pair sequences (5' to 3' direction) and primer concentrations (conc.) as used in quantitative real-time-PCR.

Accession number	Gene symbol	Gene name	Forward primer	Reverse primer	Conc. (nmol)
AV607592 XM_583951.3	<i>ACTB</i> <i>PGR</i>	Actin, β Predicted: <i>Bos taurus</i> progesterone receptor, mRNA	CGCCATGGATGATGATATTGC GAGAGCTCATCAAGGCAATTGG	AAGCCGGCCTTGACACAT CACCATCCCTGCCAATATCTTG	900/300 300/300
NM_001001443	<i>ESR1</i>	<i>Bos taurus</i> oestrogen receptor 1, mRNA	CCTCTCTCACTTCAGGCACA	ATCTCCAGCAGCAGGTCGTA	300/300
NM_174051.3	<i>ESR2</i>	<i>Bos taurus</i> oestrogen receptor 2, mRNA	GGTCAATCCATCCTACCCCT	TTCACGCCAAGGACTCTTTT	300/900

Table 2 Primary antibody sources and dilutions used for the localisation of receptor proteins in immunohistochemistry.

Primary antibody symbol	Name	Dilution	Incubation
PGR-AB	Mouse MAB, progesterone receptor Ab-8 (Clone hPRa 2 + hPRa 3)	1:250	4 °C overnight
PGR-B	Mouse MAB, progesterone receptor Ab-2 (Clone hPRa 2)	1:200	4 °C overnight
ESR1	Rabbit polyclonal antibody, oestrogen receptor Ab-17	1:50	40 min at room temperature
ESR2	Monoclonal mouse anti-human oestrogen receptor β 1	1:50	4 °C overnight

experiment. Unless otherwise stated, all washes were done twice in 0.05 M Tris-buffered saline (TBS; pH 7.7) for 5 min each, and all incubations were carried out in a humid chamber at room temperature. Four micron-thick sections of whole uterine tissue were cut from the paraffin-embedded tissue blocks, mounted on glass slides coated with 98% 3-aminopropyl triethoxysilane and dried overnight at 56 °C. One block per animal was stained, and there were five animals per treatment per time point as outlined above. From each block (i.e. animal), 15 sections were taken; from amongst these 15 sections, sections were chosen at random for testing the various antibodies. Two negative control slides (where the primary antibody was omitted) and a pre-determined positive control slide were also included in each assay.

Slides were deparaffinised by two washes in xylene and rehydrated through a series of graded alcohol steps (100 and 70%). Antigen retrieval was achieved by heating the slides in 0.01 M sodium citrate buffer (May & Baker Ltd, Essex, UK) at a pH of 6.0 for 20 min, and subsequent cooling for a further 20 min.

Endogenous peroxidase activity was neutralised by incubating the slides for 30 min with 1% hydrogen peroxide in methanol. Non-specific binding was inhibited by incubating the slides for 30 min with 2% of either normal rabbit serum (for both PGR (Laboratory Instruments and Service Centre, Ashbourne, Co., Meath, Ireland) and ESR2 (Dako Diagnostics, Cambridgeshire, UK) antibodies) or goat serum (for ESR1 antibody: Laboratory Instruments and Service Centre) in 0.05 M TBS (v/v). Primary antibodies (ESR1, PGR-AB and PGR-B sourced from Laboratory Instruments and Service Centre; and ESR2 sourced from Dako Diagnostics) were incubated at the appropriate dilution and under optimum conditions (Table 2). Slides were then washed and incubated for 45 min with the polyclonal biotinylated secondary antibodies, which was a rabbit anti-mouse at a 1:100 dilution (for PGR-AB, PGR-B and ESR2) and a goat anti-rabbit at a 1:300 dilution (for ESR1; Dako Diagnostics). The slides were washed, and avidin–biotin–HRP complex (Vectastain Elite ABC Kit, Vector Labs, Peterborough, UK) was added to the slides and they were incubated for 30 min. Following further washing, 3,3'-diaminobenzidine tetrahydrochloride chromogen substrate was added to the slides, and they were incubated for 10 min. The above-mentioned reaction was stopped by flushing the slides with distilled water, and the slides were then washed under running tap water for 7 min. This was followed by dehydration using increasing concentrations of alcohol (70 and 100%) for 5 min each and subsequent clearing in two successive changes of xylene for 10 min each. Slides were mounted using DPX (AGB Scientific Ltd, Dublin, Ireland) and observed under 10 $\times$ magnification. Using a digital

camera, four images were captured per tissue section (two images showing the LE, SGs and STR, and two images showing the DGs and MYO). Intensity of staining for all the regions was determined using Image-Pro Plus software (version 6.2, MediaCybernetics, Bethesda, MD, USA).

Statistical analyses

Differences in mRNA expression values were determined using the ΔC_t method, with expression for each gene generated relative to *ACTB*. Student's *t*-test was performed to determine significant differences in expression values for each gene of interest within treatments, across time points and within time points between treatments. All analyses for determining the effect of treatment on the intensity of localised protein were done using SAS (SAS Institute Inc., Cary, NC, USA). Parameters were checked for the assumptions underlying the ANOVA using PROC UNIVARIATE. Parameters that violated these assumptions were transformed using the appropriate λ value obtained from PROC TRANSREG. Analyses were done using PROC MIXED with day, pregnancy status, P_4 treatment and their three-way interaction as the main effects. The graphs show the least square means and their S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by Science Foundation Ireland PI grant 06/INI/B62.

Acknowledgements

The authors express gratitude to all the principal investigators, post-doctoral scientists, technical staff and all graduate students involved in the sample collection and assays as well as for their advice on various aspects of the study.

References

- Arnett-Mansfield RL, DeFazio A, Mote PA & Clarke CL 2004 Subnuclear distribution of progesterone receptors A and B in normal and malignant endometrium. *Journal of Clinical Endocrinology and Metabolism* **89** 1429–1442.

- Bazer FW, Burghardt RC, Johnson GA, Spencer TE & Wu G 2008 Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reproductive Biology* **8** 179–211.
- Bazer FW, Spencer TE, Johnson GA, Burghardt RC & Wu G 2009 Comparative aspects of implantation. *Reproduction* **138** 195–209.
- Carson-Jurica MA, Schrader WT & O'Malley BW 1990 Steroid receptor family: structure and functions. *Endocrine Reviews* **11** 201–220.
- Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans AC, Kenny DA, Roche JF & Lonergan P 2008 Effect of increasing progesterone concentration from day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reproduction, Fertility, and Development* **20** 368–375.
- Clemente M, de La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D & Lonergan P 2009 Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138** 507–517.
- De M & Wood GW 1990 Influence of oestrogen and progesterone on macrophage distribution in the mouse uterus. *Journal of Endocrinology* **126** 417–424.
- D'Haeseleer M, Cornillie P, Simoens P & van den Broeck W 2006 Localization of oestrogen receptors within various bovine ovarian cell types at different stages of the oestrous cycle. *Anatomia, Histologia, Embryologia* **35** 334–342.
- Enmark E, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M & Gustafsson JA 1997 Human estrogen receptor β -gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **82** 4258–4265.
- Forde N, Carter F, Fair T, Crowe MA, Evans AC, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P *et al.* 2009 Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biology of Reproduction* **81** 784–794.
- Garrett JE, Geisert RD, Zavy MT & Morgan GL 1988 Evidence for maternal regulation of early conceptus growth and development in beef cattle. *Journal of Reproduction and Fertility* **84** 437–446.
- Geisert RD, Fox TC, Morgan GL, Wells ME, Wettemann RP & Zavy MT 1991 Survival of bovine embryos transferred to progesterone-treated asynchronous recipients. *Journal of Reproduction and Fertility* **92** 475–482.
- Ing NH & Tornesi MB 1997 Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. *Biology of Reproduction* **56** 1205–1215.
- Kachkache M, Acker GM, Chaouat G, Noun A & Garabedian M 1991 Hormonal and local factors control the immunohistochemical distribution of immunocytes in the rat uterus before conceptus implantation: effects of ovariectomy, fallopian tube section, and injection. *Biology of Reproduction* **45** 860–868.
- Kimmins S & MacLaren LA 2001 Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. *Placenta* **22** 742–748.
- Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S & Gustafsson JA 1996 Cloning of a novel receptor expressed in rat prostate and ovary. *PNAS* **93** 5925–5930.
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β . *Endocrinology* **138** 863–870.
- Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, Quinn K & Evans A 2007 Effect of embryo source and recipient progesterone environment on embryo development in cattle. *Reproduction, Fertility, and Development* **19** 861–868.
- Mann GE, Fray MD & Lamming GE 2006 Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Veterinary Journal* **171** 500–503.
- Martin I, Torres Neto R, Oba E, Buratini J Jr, Binelli M, Laufer-Amorim R & Ferreira JC 2008 Immunohistochemical detection of receptors for oestrogen and progesterone in endometrial glands and stroma during the oestrous cycle in Nelore (*Bos taurus indicus*) cows. *Reproduction in Domestic Animals* **43** 415–421.
- Matsuzaki S, Fukaya T, Suzuki T, Murakami T, Sasano H & Yajima A 1999 Oestrogen receptor α and β mRNA expression in human endometrium throughout the menstrual cycle. *Molecular Human Reproduction* **5** 559–564.
- McNeill RE, Sreenan JM, Diskin MG, Cairns MT, Fitzpatrick R, Smith TJ & Morris DG 2006 Effect of systemic progesterone concentration on the expression of progesterone-responsive genes in the bovine endometrium during the early luteal phase. *Reproduction, Fertility, and Development* **18** 573–583.
- Mote PA, Arnett-Mansfield RL, Gava N, deFazio A, Mulac-Jericevic B, Conneely OM & Clarke CL 2006 Overlapping and distinct expression of progesterone receptors A and B in mouse uterus and mammary gland during the estrous cycle. *Endocrinology* **147** 5503–5512.
- Mulac-Jericevic B & Conneely OM 2004 Reproductive tissue selective actions of progesterone receptors. *Reproduction* **128** 139–146.
- Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP & Conneely OM 2000 Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* **289** 1751–1754.
- Mulac-Jericevic B, Lydon JP, DeMayo FJ & Conneely OM 2003 Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *PNAS* **100** 9744–9749.
- Pelletier G, Labrie C & Labrie F 2000 Localization of oestrogen receptor α , oestrogen receptor β and androgen receptors in the rat reproductive organs. *Journal of Endocrinology* **165** 359–370.
- Robinson RS, Mann GE, Lamming GE & Wathes DC 2001 Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. *Reproduction* **122** 965–979.
- Saji S, Jensen EV, Nilsson S, Rylander T, Warner M & Gustafsson JA 2000 Estrogen receptors α and β in the rodent mammary gland. *PNAS* **97** 337–342.
- Satterfield MC, Bazer FW & Spencer TE 2006 Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biology of Reproduction* **75** 289–296.
- Shughrue PJ, Lane MV, Scrimo PJ & Merchenthaler I 1998 Comparative distribution of estrogen receptor- α (ER- α) and β (ER- β) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63** 498–504.
- Spencer TE & Bazer FW 1995 Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. *Biology of Reproduction* **53** 1527–1543.
- Spencer TE & Bazer FW 1996 Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. *Endocrinology* **137** 1144–1147.
- Spencer TE, Becker WC, George P, Miranda MA, Ogle TF & Bazer FW 1995 Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. *Biology of Reproduction* **53** 732–745.
- Spencer TE, Sandra O & Wolf E 2008 Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. *Reproduction* **135** 165–179.
- Stormshak F & Bishop CV 2008 Board-invited review: estrogen and progesterone signaling: genomic and nongenomic actions in domestic ruminants. *Journal of Animal Science* **86** 299–315.
- Sunderland SJ, Crowe MA, Boland MP, Roche JF & Ireland JJ 1994 Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *Journal of Reproduction and Fertility* **101** 547–555.
- Tibbetts TA, Conneely OM & O'Malley BW 1999 Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. *Biology of Reproduction* **60** 1158–1165.
- Tsai MJ & O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Review of Biochemistry* **63** 451–486.
- Van den Broeck W, D'Haeseleer M, Coryn M & Simoens P 2002 Cell-specific distribution of progesterone receptors in the bovine ovary. *Reproduction in Domestic Animals* **37** 314–320.
- Wang H, Masironi B, Eriksson H & Sahlin L 1999 A comparative study of estrogen receptors α and β in the rat uterus. *Biology of Reproduction* **61** 955–964.
- Wang H, Eriksson H & Sahlin L 2000 Estrogen receptors α and β in the female reproductive tract of the rat during the estrous cycle. *Biology of Reproduction* **63** 1331–1340.

- Wathes DC & Hamon M** 1993 Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. *Journal of Endocrinology* **138** 479–492.
- Wathes DC, Mann GE, Payne JH, Riley PR, Stevenson KR & Lamming GE** 1996 Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes. *Journal of Endocrinology* **151** 375–393.

- Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson JA** 2000 Estrogen receptor (ER) β , a modulator of ER α in the uterus. *PNAS* **97** 5936–5941.

Received 1 March 2010

First decision 31 March 2010

Accepted 19 April 2010