The effect of season and melatonin on GnRH-induced LH secretion in oestradiol-treated orchidectomized sheep

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Abstract

The biphasic effect of oestradiol (E_2) on gonadotrope responsiveness is clearly evident in orchidectomized sheep (wethers) receiving E_2 and hourly pulses of GnRH. We hypothesized that the duration of E_2 -induced reduction in gonadotrope responsiveness differed between the breeding (November) and anoestrous (May) seasons in sheep. To test this hypothesis wethers ($n=6$ /group) were infused (i.v.) with E_2 ($2 \mu\text{g}/50 \text{ kg per h}$) and received hourly pulses of GnRH ($200 \text{ ng}/50 \text{ kg per pulse}$) or saline in May or November. The pattern of LH secretion during the 72 h infusion period was determined. Serum concentrations of LH did not differ with season in control wethers receiving vehicle alone. Similarly, continuous infusion of E_2 resulted in a 3-fold reduction in serum LH, irrespective of season. This E_2 -induced suppression of serum LH was reversed by concurrent episodic delivery of GnRH. The interval between initiation of infusion and return of pretreatment concentrations of LH was taken as a measure of the duration of E_2 -induced suppression of gonadotrope responsiveness. The duration of this E_2 -dependent response varied with season, with suppression of gonado-

trope responsiveness more prolonged ($P<0.05$) in May ($36.7 \pm 2.9 \text{ h}$) than in November ($14.3 \pm 1.1 \text{ h}$). In a companion study we examined the effect of melatonin on the duration of E_2 -induced suppression of gonadotrope responsiveness. Wethers received blank or melatonin-containing implants in March. Sixty days after implant insertion (mid-May) wethers received E_2 ($2 \mu\text{g}/50 \text{ kg per h}$) and hourly pulses of GnRH ($200 \text{ ng}/50 \text{ kg per pulse}$) or saline for 72 h. Continuous delivery of E_2 alone resulted in a 3-fold decrease in serum concentrations of LH in both control and melatonin-treated wethers. The duration of E_2 -induced suppression of gonadotrope responsiveness in animals receiving E_2 and GnRH was extended ($P<0.05$) in wethers with blank implants ($48.0 \pm 0.7 \text{ h}$), relative to the duration of suppression in melatonin-treated wethers ($14.5 \pm 1.0 \text{ h}$). Taken together these data indicate that E_2 -induced suppression of gonadotrope responsiveness is more extended during the anoestrous season. However, this seasonal effect can be reversed by continuous administration of melatonin.

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Introduction

Oestradiol (E_2) acts at hypothalamic and pituitary loci to reduce luteinizing hormone (LH) secretion (Clarke & Cummins 1984, Caraty *et al.* 1989, Scott *et al.* 1997). Oestrogenic effects at hypothalamic sites lead to a reduction in the frequency of the gonadotrophin-releasing hormone (GnRH) pulse generating system (Caraty *et al.* 1989, Karsch *et al.* 1993). Similarly, the acute response of hypophyseal targets to oestrogenic stimulation is a marked decrease in gonadotrope responsiveness, which is manifest as a reduction in the magnitude of GnRH-induced LH secretion (Clarke & Cummins 1984, Caraty *et al.* 1989). In ovariectomized sheep this oestrogen-dependent reduction in pituitary sensitivity is transient and gonadotrope responsiveness returns to pretreatment levels 12–24 h after first introduction of E_2 (Clarke & Cummins 1984, Herman & Adams 1990). A similar biphasic shift in the magnitude of

the secretory response to episodic delivery of GnRH is noted in E_2 -treated castrated male sheep (wethers) (Sakurai & Adams 1991, Sakurai *et al.* 1996).

As a photoperiod-sensitive species, sheep exhibit seasonal, or photoperiod-dependent, change in the negative feedback potency of E_2 (Legan *et al.* 1977). This is generally thought to reflect a seasonal shift in the activity of oestrogen-sensitive hypothalamic neurons that impinge on GnRH-containing neurons in the mediobasal hypothalamus (Gayrard *et al.* 1994, Havern *et al.* 1994, Lehman *et al.* 1996). The physiological response to changing photoperiod is mediated by melatonin, secretion of which is increased during the dark phase and, consequently, the duration of the nocturnal increase in the serum concentration of melatonin serves as a physiological measure of day length (Bittman *et al.* 1983, Bittman & Karsch 1984). Indeed, melatonin appears to act, at least in part, at hypothalamic sites to modulate the negative feedback

potency of E_2 (Malpaux *et al.* 1993). Although seasonal shift in the feedback potency of oestrogenic stimuli at hypothalamic sites is well documented, recent work indicates that gonadotrope responsiveness may also vary with season or photoperiod (Xu *et al.* 1992, Tilbrook *et al.* 1993).

In the studies reported here we used wethers treated with E_2 and episodic GnRH to test the hypothesis that the duration of oestrogen-dependent suppression of gonadotrope responsiveness varies with season. We further hypothesized that continuous treatment with melatonin would reverse this season-dependent shift in the negative feedback potency of E_2 at hypophyseal sites.

Materials and Methods

Animals

Spring- or fall-born Suffolk lambs were castrated within 2 weeks of birth. The wethers were housed in an open-sided barn under natural lighting and were afforded free access to water and alfalfa pellets supplemented with cereal grains and vitamin and mineral premix. They were 6–8 months of age (weight 60–65 kg) at the time of experimentation. All experimental procedures involving the use of animals were conducted in accordance with National Institutes of Health (NIH) Guidelines and were reviewed and approved by the Animal Use and Care Committee for the University of California.

Cannulation

Before experimentation, two polyethylene cannulae (Intramedic PE 190, Clay Adams, Parsippany, NJ, USA) were inserted into the left jugular vein to serve as hormone delivery cannulae (E_2 or GnRH). A third cannula (Intramedic PE 190), inserted into the contralateral vein, was used for blood collection. All cannulae were passed through a protective plastic tubing sheath to the exterior of the animal holding area. Animals were freely mobile at the end of a 1 m lead.

Hormone delivery

Cannulae for the delivery of E_2 , GnRH or the appropriate vehicle were connected to syringes placed in Harvard infusion pumps (Model 2265, Harvard Bioscience, South Natick, MA, USA). Oestradiol (Sigma Chemical Co., St Louis, MO, USA; 2 µg/ml) in 10% ethanol-saline (E_2 delivery vehicle; EV), or EV alone, was delivered as a continuous infusion.

An episodic pattern of delivery of GnRH (Peninsula Laboratories, Belmont, CA, USA; 200 ng/50 kg per h pulse) in saline, or saline alone, was effected using an infusion pump connected to an automatic timer

Figure 1 Experimental protocol. The effect of season and melatonin on the duration of E_2 -dependent suppression of gonadotrope responsiveness was assessed in wethers receiving continuous E_2 (2 µg/50 kg per h) and circhoral GnRH (200 ng/50 kg per h pulse) for 72 h. The duration of the oestrogenic response was assessed during the breeding (November) and anoestrous (May) seasons. In addition, gonadotrope responsiveness was assessed in May in wethers receiving control (Blank) or melatonin-containing (Melatonin) implants 60 days earlier.

(Chroncontrol, Lindburg Enterprises, San Diego, CA, USA) that was programmed to deliver a 5 min pulse (1 ml volume) once each hour. During episodic administration of GnRH, blood samples were collected immediately before GnRH delivery.

Melatonin-containing implants were prepared using a modification of the procedure used by Diskin & Niswender (1989) to prepare progesterone implants. Briefly, 5 g crystalline melatonin (Sigma Chemical Co.) were combined with 20 ml Silastic Medical Adhesive (Type A; Dow Corning, Midland, MI, USA). The melatonin-adhesive mixture was allowed to harden after injection into a tubular mold (6.4 mm internal diameter). The melatonin-containing silastic was removed from the mold 72 h after injection and cut into 5 cm lengths. Each implant was estimated to contain 500 mg melatonin. The implants were washed overnight in sterile saline before use. Melatonin-free, or blank, implants were prepared by deleting melatonin from the protocol described above. Serum concentrations of melatonin during exposure to sunlight (sample collection during the early afternoon) were 22.8 ± 3.3 and 119.6 ± 11.1 pg/ml in sheep carrying blank and melatonin-containing implants respectively.

Experiment 1

The effect of season on the duration of the E_2 -dependent suppression of gonadotrope responsiveness was assessed during the breeding (November) and anoestrous (May) seasons using spring-born ($n=18$) or fall-born ($n=18$) wethers respectively (Fig. 1). Fall-born wethers were assigned at random to one of three treatment groups ($n=6$ /group) in mid-May. Continuous infusion of EV (group 1) or E_2 (2 µg/50 kg per h; groups 2 and 3) was

initiated at time 0 and continued for 72 h. Episodic administration of saline (groups 1 and 2) or GnRH (200 ng/50 kg per h pulse; group 3) was also initiated at time 0 and continued throughout the infusion period. Blood samples were collected at 2 h intervals during the first 24 h of infusion and at 4 h intervals thereafter. Serum was harvested and stored frozen at -20°C for later endocrine analysis. This experimental protocol was repeated in November using spring-born wethers.

Experiment 2

The effect of melatonin-containing implants on the duration of the E_2 -induced suppression of gonadotrope responsiveness in wethers was assessed during the anoestrous season (May; Fig. 1). Briefly, 28 wethers were assigned at random to one of four treatment groups. Blank (groups 1 and 2; $n=6/\text{group}$) or melatonin-containing (groups 3 and 4; $n=8/\text{group}$) implants were inserted s.c. in mid-March. Oestrogen-dependent change in gonadotrope responsiveness was assessed in mid-May, 60 days after implant insertion. Animals in all groups received E_2 ($2\text{ }\mu\text{g}/50\text{ kg per h}$) as a continuous infusion for 72 h. Animals in groups 1 and 3 received hourly pulses of saline during the infusion period, while animals in groups 2 and 4 received episodic GnRH (200 ng/50 kg per h pulse) throughout the 72 h period of infusion. Blood samples were collected, processed and stored as described above.

Endocrine analysis

Serum concentrations of LH, E_2 and melatonin were determined using previously validated procedures (Adams *et al.* 1975, English *et al.* 1986, Sakurai *et al.* 1992). The LH reference standard (NIAMDD-oLH-25) was a gift of the National Hormone and Pituitary Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD; Baltimore, MD, USA). In all cases intra- and interassay coefficients of variation were less than 10%.

Statistical analysis

The significance of treatments was assessed by ANOVA or, where appropriate, ANOVA for repeated measures (Gill 1978). Differences between treatment means were tested for significance using Duncan's multiple range test. Data are presented in the text and figures as means $\pm$ S.E.M.

Results

Serum concentrations of E_2 were increased to 11.5 ± 0.6 and $11.2 \pm 0.2\text{ pg/ml}$ within 2 h of initiation of E_2 infusion in May and November respectively, and the serum concentration of E_2 remained at that level for the duration

Figure 2 Serum concentrations of LH during the breeding (November) and anoestrous (May) seasons in wethers ($n=6/\text{group}$) receiving continuous oestradiol (E_2 ; $2\text{ }\mu\text{g}/50\text{ kg per h}$) and circhoral pulses of saline (○) or GnRH (200 ng/50 kg per h pulse; ●) for 72 h.

of E_2 delivery. Similarly, in experiment 2 serum concentrations of E_2 were increased to $10.2 \pm 0.3\text{ pg/ml}$ 2 h after initiation of E_2 infusion and remained at this level for the duration of the infusion period. In contrast, serum concentrations of E_2 were not detected ($<0.6\text{ pg/ml}$) in wethers receiving vehicle alone.

Experiment 1

Mean serum concentration of LH did not vary with season (6.4 ± 1.3 and $5.7 \pm 0.6\text{ ng/ml}$ in May and November respectively) in wethers prior to treatment (Fig. 2). Similarly, serum concentrations of LH did not differ ($P>0.05$) from the pretreatment levels in control wethers receiving EV and hourly pulses of saline for 72 h in May ($5.9 \pm 0.4\text{ ng/ml}$) and November ($5.4 \pm 0.4\text{ ng/ml}$).

Continuous infusion of E_2 resulted in a rapid and marked suppression of LH in both the breeding and anoestrous seasons (Fig. 2). In both seasons serum

concentrations of LH were significantly ($P<0.05$) decreased 2 h after initiation of E_2 delivery (4.2 ± 0.4 and 3.3 ± 0.7 ng/ml in May and November respectively) and nadir concentrations of LH were evident after 6 h of E_2 administration (2.9 ± 0.3 and 1.9 ± 0.4 ng/ml in May and November respectively). In the absence of supplemental GnRH, serum concentrations of LH were maintained at nadir levels for the remainder of the E_2 delivery period.

The decrease in serum LH induced by E_2 infusion was evident even in wethers receiving concurrent circhoral pulses of exogenous GnRH. However, episodic delivery of GnRH eventually returned the serum concentration of LH to the pretreatment level. The duration of the E_2 -dependent decrease in gonadotrope responsiveness varied with season, with the interval from initiation of infusion to re-establishment of pretreatment concentrations of LH more extended ($P<0.05$) in May (36.7 ± 2.9 h) than November (14.3 ± 1.1 h).

Experiment 2

Mean serum concentration of LH in May did not vary in wethers with blank (7.8 ± 0.6 ng/ml) or melatonin-containing (7.8 ± 0.8 ng/ml) implants (Fig. 3). Initiation of E_2 infusion resulted in a rapid and marked suppression of LH and nadir concentrations of LH (evident after 6 h of E_2 administration) did not differ in blank- or melatonin-treated wethers (2.8 ± 0.2 and 3.0 ± 0.3 ng/ml respectively). In the absence of supplemental GnRH, serum concentrations of LH were maintained at nadir levels for the remainder of the E_2 delivery period.

The E_2 -dependent decrease in serum LH was also evident in wethers receiving E_2 and concurrent hourly pulses of GnRH and the magnitude of the oestrogen-dependent decrease in serum concentrations of LH did not vary ($P<0.05$) with melatonin treatment. After the initial decrease, serum concentrations of LH were returned to pretreatment concentrations in wethers receiving E_2 and episodic GnRH in combination. However, the duration of the E_2 -dependent decrease in gonadotrope responsiveness was significantly affected by melatonin treatment. The interval from initiation of infusion to re-establishment of pretreatment concentrations of LH was markedly reduced ($P<0.05$) in melatonin-treated wethers (14.5 ± 1.0 h), relative to the duration of suppression noted in wethers with blank implants (48.0 ± 0.7 h).

Discussion

The seasonal shift in the negative feedback potency of E_2 in seasonally breeding species, such as sheep, is well documented (Legan *et al.* 1977). Most studies suggest that the E_2 -dependent decrease in LH secretion evident during the non-breeding season reflects oestrogenic action at hypothalamic loci to reduce the frequency of episodic

Figure 3 Serum concentrations of LH during the anoestrous (May) season in wethers treated with control (Blank; $n=6$ /group) or melatonin-containing (Melatonin; $n=8$ /group) implants and receiving continuous oestradiol (E_2 ; $2 \mu\text{g}/50 \text{ kg per h}$) and circhoral pulses of saline (○) or GnRH ($200 \text{ ng}/50 \text{ kg per h pulse}$; ●) for 72 h.

GnRH secretion (Karsch *et al.* 1993, Viguie *et al.* 1995). Indeed, pulsatile delivery of low amplitude pulses of GnRH reactivates follicular development and leads to the ovulatory surge of the gonadotrophins in sheep during the period of seasonal anoestrus (McLeod *et al.* 1982).

However, action at hypothalamic sites may not account, in full, for the suppressive effects of E_2 on LH secretion. In this regard it is important to note that the onset and intensity of E_2 -dependent inhibition of LH secretion is enhanced when the oestrogenic stimulus is applied systemically, rather than directly to hypothalamic sites (Gallegos-Sanchez *et al.* 1996, 1997). In addition, several reports indicate that gonadal steroids act directly at hypophyseal loci to alter gonadotrope sensitivity. The direct effect of E_2 on gonadotrope responsiveness is clearly evident in wethers receiving E_2 and hourly pulses of GnRH. In this treatment paradigm serum concentrations of LH are markedly decreased 6 h after first introduction of E_2 . However, during the breeding season pretreatment

concentrations of LH are restored 12 to 24 h after beginning E_2 infusion in wethers receiving concurrent episodic GnRH stimulation (Sakurai & Adams 1991, Sakurai *et al.* 1996). A similar biphasic pattern of LH secretion is noted in E_2 -treated ovariectomized sheep made deficient in endogenous GnRH by hypothalamic–pituitary disconnection (Clarke & Cummins 1984, Clarke *et al.* 1988) or passive immunization (Caraty *et al.* 1984, Herman & Adams 1990) and receiving hourly pulses of GnRH or a GnRH agonist. Biphasic effects of E_2 on gonadotrope responsiveness are also evident *in vitro* (Frawley & Neill 1984, Ortmann *et al.* 1988). Collectively, these observations indicate that the negative feedback effects of E_2 reflect oestrogenic actions directed, at least in part, at hypophyseal loci.

In the studies presented here we used wethers receiving E_2 and GnRH to examine the effect of season and melatonin on the duration and intensity of E_2 -dependent suppression of gonadotrope responsiveness. These results indicate that physiological concentrations of E_2 act acutely at hypophyseal loci to suppress gonadotrope responsiveness and GnRH-induced LH secretion. Interestingly, the duration of this oestrogen-dependent response varies with season. Indeed, the pituitary-specific suppression of LH is approximately three times longer during the anoestrous season. A similar seasonal shift in gonadotrope responsiveness is evident in wethers receiving exogenous testosterone. In this model, GnRH-induced LH (Xu *et al.* 1992) and follicle-stimulating hormone (Tilbrook *et al.* 1993) secretion varies with season, with significantly reduced gonadotrope responsiveness during the non-breeding season.

Oestrogen is postulated to act at hypothalamic sites in a season- or photoperiod-specific manner to alter the activity of regulatory neurons that impinge on the GnRH-containing neuronal system (Gayrard *et al.* 1994, Havern *et al.* 1994, Lehman *et al.* 1996, Skinner & Herbison 1997). In contrast, the mechanism of E_2 -induced decrease in pituitary responsiveness has not been determined. However, oestrogen stimulation is associated with a rapid and marked increase in pituitary concentrations of GnRH receptor (Clarke *et al.* 1988, Sakurai & Adams 1991). Thus it seems likely that the reduction in gonadotrope responsiveness noted during acute oestrogenic stimulation is not related to receptor concentration but, rather, may be mediated by a decoupling of the GnRH receptor from critical second messenger systems.

The seasonal control of reproductive function in sheep is mediated by photoperiod and the duration of elevated melatonin secretion associated with the nocturnal phase (Legan *et al.* 1977, Bittman *et al.* 1983, Bittman & Karsch 1984). The physiological response characteristic of short day length is evident even during periods of extended day length when exogenous melatonin is provided 6–8 h before the actual onset of darkness (Kennaway *et al.* 1982, English *et al.* 1986, Poulton *et al.* 1987). Insertion of

constant release implants containing melatonin has a similar effect (English *et al.* 1986, Poulton *et al.* 1987), leading to the suggestion that continuous release implants induce the physiological response characteristic of short day length (English *et al.* 1986, O'Callaghan *et al.* 1991). Our results are consistent with this postulate since short day length or constant melatonin during periods of long day length induced the same response, a reduction in the duration of the negative feedback action of E_2 at hypophyseal loci.

In contrast to the acute oestrogenic response, GnRH-induced LH secretion is restored during more prolonged E_2 stimulation. The mechanism underlying this recovery of response capacity has not been resolved. However, E_2 -dependent augmentation of tissue concentrations of GnRH receptor may play an important role in this process. Indeed, physiological concentrations of E_2 increase tissue concentrations of GnRH receptor in pituitary tissue of sheep, with the maximal augmentation evident after 12 to 24 h of oestrogen stimulation (Clarke *et al.* 1988, Sakurai & Adams 1991). Although basal concentrations of GnRH receptor do not vary with season or melatonin status (Sakurai *et al.* 1995, T E Adams & H Sakurai, unpublished observations) we postulate that season and melatonin status may affect the temporal pattern of E_2 -dependent increase in GnRH receptor, with this oestrogenic response delayed during the anestrus season.

Although the specific site of action of melatonin was not resolved in these studies, melatonin may act at both hypothalamic and hypophyseal loci to influence the negative feedback potency of E_2 . The importance of action at hypothalamic sites is suggested by the observation that direct delivery of melatonin to the mediobasal hypothalamus reduces the negative feedback potency of E_2 in sheep maintained under a long day photoperiod (Malpoux *et al.* 1993). However, melatonin may also exert effects directly at hypophyseal sites. Indeed, hypophyseal tissue, particularly the pars tuberalis, is richly invested with melatonin receptors (Bittman & Weaver 1990, Helliwell & Williams 1994, Reppert *et al.* 1994) and is sensitive to direct stimulation by melatonin (Hazlerigg *et al.* 1993). Direct action of melatonin at hypophyseal sites is also suggested by the recent observation that photoperiod-dependent cycles of prolactin secretion persist after physical disconnection of the hypothalamus and pituitary in male sheep (Lincoln *et al.* 1996).

Collectively the data presented here indicate that E_2 acts at hypothalamic and hypophyseal loci to inhibit LH secretion. The oestrogenic effect at hypophyseal sites is manifest as a transient decrease in the magnitude of GnRH-induced LH secretion. Interestingly, the duration of this E_2 -induced suppression of gonadotrope responsiveness fluctuates with the season, with oestrogen-dependent suppression at pituitary sites particularly extended during the non-breeding season. Constant-release melatonin implants reverse the oestrogen-dependent suppression

of responsiveness characteristic of long day length photoperiods. The rapid onset of the negative feedback response at pituitary loci, in combination with the seasonal shift in the duration of the oestrogenic response, suggests that oestrogenic action at both hypophyseal and hypothalamic sites may act in concert to induce the profound suppression of LH secretion characteristic of the anoestrous period.

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