Gonadotropin inhibitory hormone (GnIH) prevents the 'priming' effect of estradiol-17β

Gonadotropin inhibitory hormone (GnIH) mempengaruhi efek puncak estradiol-17β

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Abstract

GnIH inhibits GnRH-stimulated gonadotropin secretion in the sheep by action on the pituitary gonadotropes. Estradiol-17β (E_2) also acts at the level of the pituitary gonadotrope to exert negative and positive feedback effects on gonadotropin secretion. The positive effect facilitates the estrogen-induced surge in gonadotropin releasing hormone (GnRH) and LH. This study was undertaken to determine whether this 'priming' effect of E_2 is prevented by GnIH. Cultured pituitary cells were treated with GnRH or vehicle every 4 h for 24 h with and without GnIH and E_2 treatment. GnRH stimulated (LH) and follicle stimulating hormone (FSH) secretion was increased by E_2 treatment. The effect of E_2 was overcomes by the inhibitory effect of GnIH. We conclude that GnIH may counteract the priming effect of E_2 on pituitary gonadotropes.
Key words: Gonadotropin, gonadotrope, gonadotropin inhibitory hormone, estradiol

Abstrak

Gonadotropin inhibitory hormone (GnIH) terbukti mampu menghambat pelepasan gonadotropin dari pituitary domba. Estradiol-17β (E_2) memiliki kemampuan baik menghambat maupun menstimulasi sekresi gonadotropin. Adanya stimulasi E_2 tampak pada terjadinya puncak sekresi dari GnRH dan LH saat terjadinya estrus. Penelitian ini ditujukan untuk melihat apakah pacuan dari E_2 pada saat terjadinya puncak estrus dapat dipengaruhi oleh GnIH. Kultur sel pituitari dari domba betina dewasa digunakan dalam penelitian ini, dilakukan perlakuan dengan atau tanpa GnRH selama 4 sampai 24 jam, bersamaan dengan pemberian GnIH dan E_2. Sekresi gonadotropin (LH dan FSH) yang distimulasi oleh GnRH terbukti ditingkatkan oleh adanya E_2, sementara efek stimulasi oleh E_2 ini mampu ditahan oleh GnIH. Hal ini membuktikan adanya peran GnIH sebagai pengontrol negatif bagi pacuan E_2 dalam sel-sel di pituitari pada saat terjadinya estrus pada domba betina.
Kata kunci: gonadotropin, pituitari, GnIH, estradiol

Introduction

Gonadotropin releasing hormone (GnRH) provides the primary stimulus for the reproductive axis, through action on the pituitary gonadotropes and gonadotropin inhibitory hormone (GnIH) counteracts this effect (Clarke, *et al.,* 2008; Smith and Clarke, 2010).Our studies in sheep have presented convincing evidence that GnIH is an hypophysiotropic factor in this species. GnIH-expressing cells are located in the periventricular zone of the ventral paraventricular nucleus and the dorsomedial nucleus and these cells project to the external zone of the median eminence (Clarke, *et al.*, 2008). Although there is no evidence to date that GnIH is secreted into hypophysial portal blood, the peptide has a potent effect on the gonadotropes through inhibition of the release of intracellular calcium caused by GnRH and it also reduces amplitude of pulsatile LH secretion when administered intravenously to OVX ewes (Clarke, *et al.,* 2008).

Prior to the preovulatory surge in LH the response of the gonadotropes to GnRH is increased. This is due to a 'priming' effect of GnRH as well as E_2. GnRH self-priming was shown in a series of studies (de Koning, *et al.,* 2001; Evans, *et al.,* 1984; Waring and Turgeon, 1980; Veldhuis, *et al.,* 1986). Increased GnRH pulse frequency during the follicular phase of the estrous cycle increases the number of gonadotropes expressing estrogen receptor α (ERα) (Clarke, *et al.,* 2005), sensitising the cells to the effect of E_2 and increasing responsiveness to GnRH, in a variety of species including rats, sheep, and women (Arimura *et al.,* 1971; Clarke and Cummins, 1984; Yen *et al.,* 1973). This sensitisation is seen in cultures of in sheep, bovine and rat pituitary cells (Drouin *et al.,* 1976; Huang and Miller, 1980), at least part of which is due to upregulation of the GnRH receptor (GnRH-R) (Adams and Spies, 1981; Marian, *et al.,* 1981; Nett, *et al.,* 1984). The effect of E_2 on GnRH-R expression and binding is seen in female gonadotropes in the follicular phase of the estrous cycle (Cowley, *et al.,* 1998) and is also shown in models where E_2 acts directly on the pituitary gland *in vivo* (Clarke, *et al.,* 1988). E_2 also acts on the gonadotropes to alter post-receptor GnRH signalling pathways, such as calcium flux (Heyward, *et al.,* 1995), levels of diacylglycerol (DIG) (Chang, *et al.,* 1988), protein kinase C (PKC) (Drouva, *et al.,* 1990; Stojilkovic, *et al.,* 1988) and cyclic adenosine monophosphate (cAMP) (Kamel and Krey, 1983; Tang, *et al.,* 1982). E_2 also increases the function of the inwardly rectifying potassium current in gonadotropes (Cowley, *et al.,* 1999). E_2 also causes migration of the LH-containing granules to the plasma membrane of the gonadotropes in preparation for the massive secretory event that occurs during the surge (Thomas and Clarke, 1997). The rapid non genomic effect of estrogen has been demonstrated in ovine pituitary gonadotropes. Both *in vivo* and *in vitro* effect of E_2 are exhibited by phosphorylation of ERK-1/2 and cAMP-responsive element-binding protein (CREB) (Iqbal, *et al.,* 2009).

The question arises as to whether the negative effect of GnIH may counter the priming effects of E_2. In order to address this, we have developed an *in vitro* model that allows study of the response to GnRH over 16h, which is the time-frame over which E_2 acts to cause a positive feedback effect (Sari, *et al.,* 2009). Herein, we describe studies to dissect the effects of GnRH in combination with E_2 and/or GnIH and we show that GnIH may counteract the priming effect of E_2.

Methodology

Animals

Adult ovariectomised (OVX) ewes of the Corriedale breed were of similar age (5 years) and body weight and were sourced from the Monash University Sheep Facility, Werribee, Victoria. All experiments were conducted according to the guidelines established by the Australian Prevention of Cruelty to Animals Act 1986 and the study was approved in advance by the Monash University Animal Ethics Committee.

Pituitary collection and preparation

Animals were euthanized by an overdose of sodium pentobarbital (Lethabarb; Virbac, Peakhurst, NSW, Australia) and pituitaries were collected into Dulbecco's modified Eagle's medium (DMEM) (Thermo Electron Corp., Melbourne, Australia) containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich Inc, St. Louis, USA). Pituitaries were diced and minced and cells were then enzymatically dissociated and dispersed by agitation for 35 min at 37 °C in 10 ml of phosphate buffered saline (PBS) (Gibco Invitrogen Corp., Auckland, NZ) containing 0.1% BSA, collagenase-I (Wothington Biochemical Corp., Lakewood, USA) (3mg/mL), trypsin inhibitor (0.5mg/mL), DNase-I (20μg/mL), hyaluronidase-II (1mg/mL) and pancreatin (1.25mg/mL)) (all from Sigma-Aldrich Inc, St. Louis, USA). The cells were passed through fine mesh and centrifuged, then resuspended in 0.1% BSA/PBS. Yield was approximately 80-90 x 10^6 cells/pituitary with >95% viability (trypan blue exclusion test). For culture, the pelleted cells were resuspended in DMEM containing 10% fetal calf serum, streptomycin and antimycotic (all from Gibco Invitrogen Corp., Auckland, NZ).

Effect of GnIH and E_2 on gonadotropin secretion

For each experiment, pituitaries from 2 OVX ewes were collected and the cells from both animals were mixed. Cultures of $1x10^6$ cells/well were established in 1 mL culture medium and the medium was changed daily for 2 days prior to experimentation. On the day prior to experimentation and throughout experimentation, the cells were incubated in medium free of phenol red.

On day 3 the following treatments were applied:-

- GnRH (Auspep, Parkville, Vic., Australia) at doses of 10^{-9}, $10^{-10}$and 10^{-12}M or vehicle;
- E_2 (Intervet, Artarmon, NSW, Australia) at doses of 10^{-8}- 10^{-12}M or vehicle;
- GnRH (10^{-12} or 10^{-10}M) plus E_2 (10^{-10} or 10^{-8}M) or vehicle;
- Treatments that were the same as above with and without treatment GnIH (Auspep, Parkville, Vic., Australia) at a dose of 10^{-9}M.

At each timepoint (0, 4, 8, 12 and 16h) after commencement of GnRH/ E_2/GnIH/vehicle treatment, medium was removed and replaced with fresh medium containing the relevant treatment. The samples were stored at -20 C until assayed for LH and FSH by radioimmunoassay. The experiments were replicated in 3 separate cultures.

Radioimmunoassays

Samples were diluted 1:20 for assay. LH concentrations were measured by radioimmunoassay as described by Lee, *et al.,* (1976) using ovine NIH-oLH-S18 as the standard. The average sensitivity of the LH assays was 0.1ng/mL and the intra-assay coefficient of variation (CV) was less than 10% over the range of 0.5-9.4 ng/mL. Inter-assay CV was 10.4%. The method of Bremner, *et. al.,* (1980) was used for FSH measurement using ovine NIAMDD-oFSH-RP-1 as the standard. The average sensitivity of the FSH assays was 0.1ng/mL and the intra-assay coefficient of variation (CV) was less than 10% over the range of 0.2-15.2 ng/mL. Inter-assay CV was 5.4%.

Statistical Analysis

Data are presented as mean (±SEM). Statistical analysis was performed using SPSS (version 15.0, SPSS Inc, Chicago, IL, USA) using repeated measures ANOVA and Scheffe's method as a *post-hoc* test.

Results and Discussion

Repeated treatment with GnRH at 4h intervals at the doses 10^{-9}M and 10^{-10} M (but not 10^{-12} M) stimulated LH and FSH secretion (Fig 1, panels A and B). Stimulation of LH (Fig 1, panel A) was more marked than that of FSH (Fig 1, panel B).

At doses 10^{-9} and 10^{-8}M (but not at lower doses), treatment with E_2 alone increased LH secretion (Fig 1, panel C, $P<0.05$; $P<0.01$). At these doses, E_2 reduced FSH secretion (Fig.1, panel D, $P<0.05$). Based on the results of the above treatments, doses of GnRH and E_2 were chosen that might reveal the priming effect of E_2 on GnRH action. We used 10^{-10} and 10^{-8}M doses of E_2 with 10^{-10}M and 10^{-12} doses of GnRH alone or in combination. At a dose of 10^{-10}M GnRH, the priming effect of E_2 on LH response was seen at the 8h time point only and only with 10^{-8}M E_2 (Fig 2A; $P<0.01$). Interestingly, a priming effect on FSH secretion was seen at both doses of E_2 and at all time points (Fig 2B; $P<0.05$). At a dose of 10^{-12}M GnRH, there was no overt priming effect of E, since the combined effect of GnRH plus E_2 was not greater than that of E_2 alone (Fig 3, panels A and B). With a dose of 10^{-10}M GnRH, GnIH (10^{-9}M) reduced the secretion of LH and FSH (Fig.2, panels A and B). With combined GnRH and E_2 (10^{-8}M), GnIH prevented the priming effect of E_2 on the secretion of both LH and FSH. This effect was seen at all time points.

Using an *in vitro* model, we have shown E_2 priming of the response of pituitary gonadotropes to GnRH and this is apparent over the time course that is relevant to the LH-surge generation obtained by E_2 treatment of OVX ewes (Clarke, *et al.,* 2005). This provides a means by which we can examine factors that might influence this priming effect and will also permit future studies on the sub-cellular signalling events underlying this phenomenon.

GnRH at the doses 10^{-9}M and 10^{-10}M (but not lower doses) stimulated LH and FSH secretion. As shown in other culture systems (Huang and Miller, 1980) and *in vivo* (Clarke, *et al.,* 1984), GnRH elicits a greater incremental rise in secretion of LH than FSH, but the effect on both gonadotropins is sustained throughout 16h, with 4 hourly replacement of the medium with GnRH. Interestingly, a dose-response effect on LH secretion is seen at 8h, but this is lost at later time points, where effects of 10^{-10} M GnRH are as effective as 10^{-9}M GnRH. The priming effect of E_2 on GnRH stimulated LH and FSH secretion is apparent only at a sub-maximal (10^{-10}M) dose of GnRH and only with relatively high E_2. Further studies may allow us to determine the relative effects of GnRH and E_2 on sub-cellular signalling in this system,

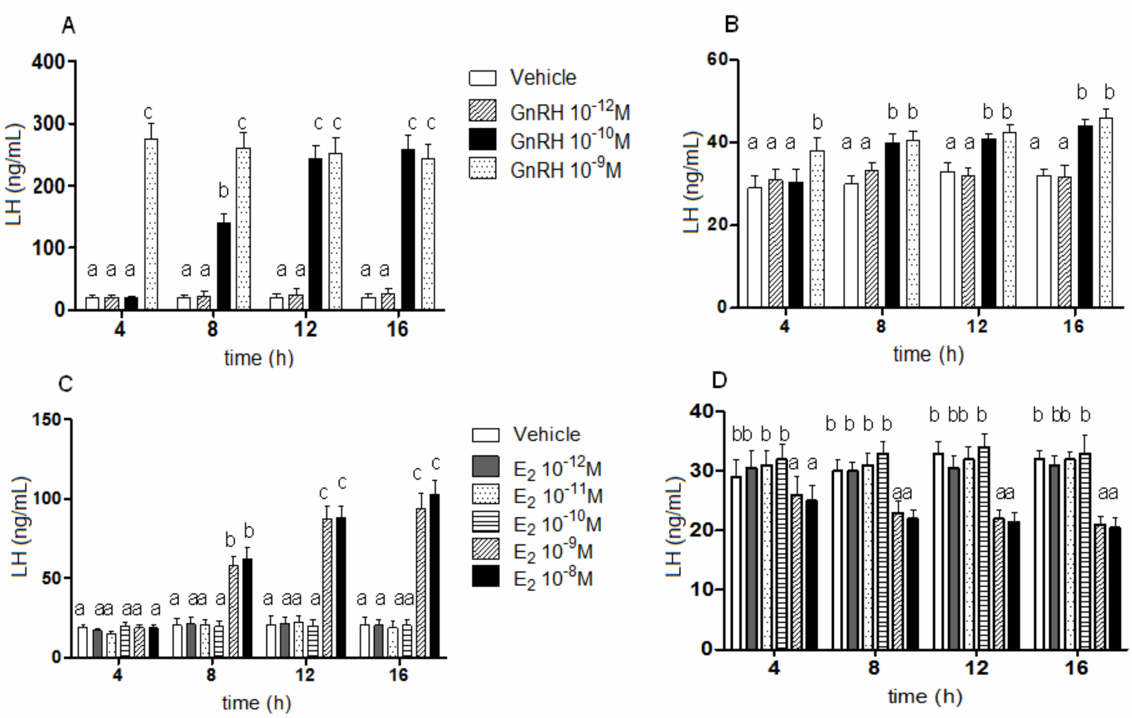

Figure 1. Effects of either GnRH or E2 across 4-16h on LH and FSH secretion from OVX ewe pituitary cells in primary culture. Medium was removed every 4h and replaced with medium containing the appropriate dose of GnRH or E2. Effects of dose of GnRH are shown in panels A and B. For panels A and B, a vs. b, $P<0.05$; b vs. c, $P<0.05$; a vs. c, $P<0.01$. Panels C and D show the effect of treatment with E2 alone on LH and FSH secretion. The data are means (±SEM) of 3 cultures. For Panels C and D, a vs. b, $P<0.05$; b vs. c, $P<0.05$; a vs. c, $P<0.01$.

using primary pituitary cells. The priming effect of E_2 is most readily apparent at the 8h timepoint in relation to LH secretion, but is seen at all time points for FSH secretion.

In a physiological setting, the gonadotropes would not be exposed to E_2 alone, but a response to the steroid hormone will be useful for the study of signalling, especially rapid E_2 effects. Earlier studies have shown that chronic treatment bovine and rat pituitary cell culture with E_2 stimulates basal LH release (Kamel and Krey, 1983). In the present study, we found that doses of 10^{-8}M or 10^{-8}M increases basal LH secretion from ovine gonadotropes. On the other hand, treatment with E_2 alone reduces FSH secretion, which is consistent with other studies (Huang and Miller, 1980; Miller and Huang, 1985). Whereas the effect of E_2 in the follicular phase of the estrous cycle is to stimulate LH secretion, FSH levels fall (Goodman, *et al.,* 1982), but this could be due to the effect of inhibin (Clarke, *et al.,* 1986). Our culture system will allow us to determine signalling effects of GnRH and E_2 but it will be difficult to discriminate between those relevant to LH secretion vs FSH secretion. Treatment of ovine pituitary cell culture with an antibody to activin B reduced FSH secretion without affecting LH secretion. This effect was accompanied by the reduction in activin β_B mRNA expression, suggesting that the inhibitory effect of E_2 on FSH secretion from the pituitary gonadotropes may be mediated by activin (Baratta, *et al.,* 2001).

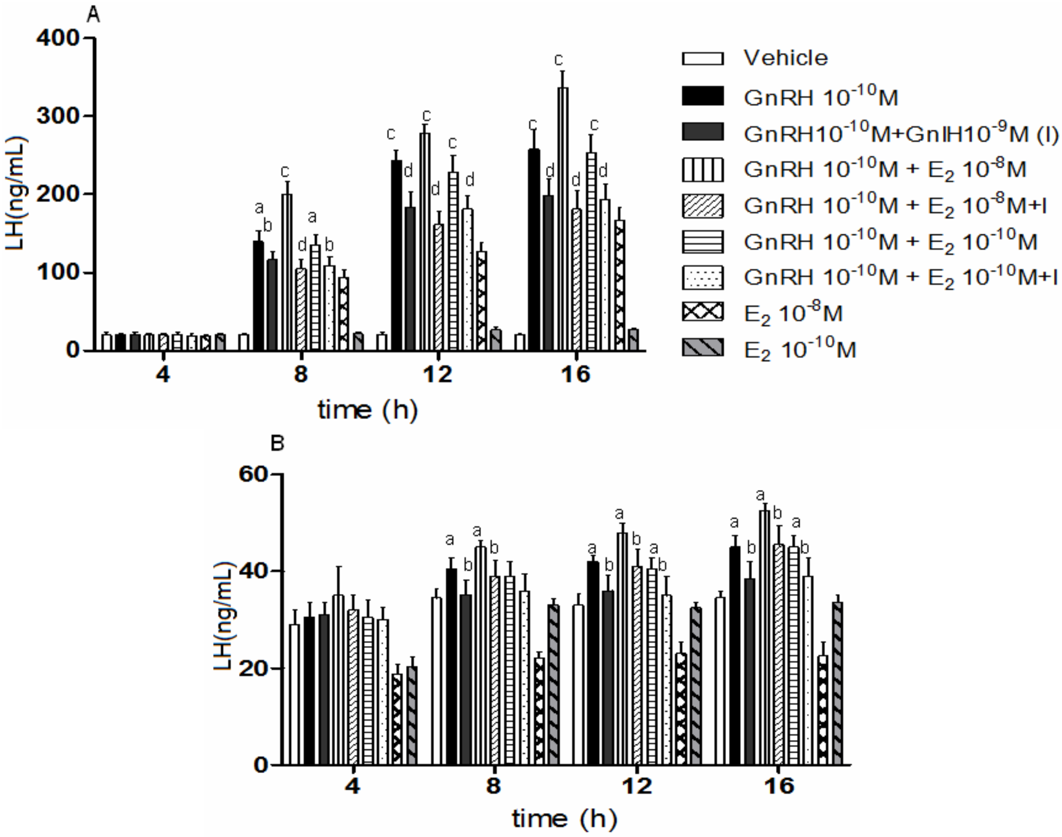

Figure 2. Inhibitory effects of GnIH (10-9M) on either GnRH (10-10M) alone or in combination with E2 (10-8 and 10-10M) across 4-16h on LH and FSH secretion (panel A and B, respectively) from OVX ewe pituitary cells in primary culture. Medium was removed every 4h and replaced with medium containing the appropriate dose of GnRH or E2. For panels A and B, a vs. b, $P<0.05$; c vs. d, $P<0.01$. The data are means (±SEM) of 3 cultures.

Treatment effects of E2 alone are the same as those shown in Fig 1 and are presented here for comparison.

The model we used indicates a time when positive feedback mechanism is operative, showing the effect of E_2 to sensitize cells to respond to GnRH (Clarke and Cummins, 1984). This model allows examination of the effect of GnIH in the positive feedback effect of E_2. As mentioned in the introduction, E_2 affects a number of signalling pathways, in gonadotropes. One or more of these may relate to priming by E_2 and this is unmasked with a submaximal dose of GnRH (10^{-10}M) and a dose of 10^{-8}M E_2. At these doses, an effect is seen on LH secretion and the effect of E_2 on FSH secretion is the opposite of that seen with E_2 alone. In this setting, GnIH reduces the stimulatory effect of GnRH, consistent with our earlier studies (Clarke, *et al.,* 2008) and also prevents the priming effect of E_2. In ovine brain, the expression of GnIH mRNA is also reduced in the late-follicular phase of the ewe estrous cycle. GnIH treatment also eliminates pulsatile LH secretion in the mid-follicular phase and blocks LH surge (Sari, *et al.,* 2009). These results indicate that the reduction of GnIH expression in the follicular phase of the ewe is permissive for LH surge.

The combination of E_2 and GnRH at the time of positive feedback involves a range of priming events at the sub-cellular level,as indicated in the Introduction. As to which of these events is compromised by the inhibitory effect of GnIH remains to be determined and is the focus of ongoing studies.

Conclusion

GnIH is an hypophysiotropic hormone which is able to prevent the priming effect of E_2 on gonadotropes in the period leading up to a surge in LH secretion.

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