Effect of phytoestrogens on basal and GnRH-induced gonadotropin secretion

Sergio A Arispe, Betty Adams and Thomas E Adams

Department of Animal Science, University of California, Davis, One Shields Avenue, Davis, California 95616, USA

Correspondence should be addressed to T E Adams
Email
teadams@ucdavis.edu

Abstract

Plant-derived estrogens (phytoestrogens, PEs), like endogenous estrogens, affect a diverse array of tissues, including the bone, uterus, mammary gland, and components of the neural and cardiovascular systems. We hypothesized that PEs act directly at pituitary loci to attenuate basal FSH secretion and increase gonadotrope sensitivity to GnRH. To examine the effect of PEs on basal secretion and total production of FSH, ovine pituitary cells were incubated with PEs for 48 h. Conditioned media and cell extract were collected and assayed for FSH. Estradiol (E_2) and some PEs significantly decreased basal secretion of FSH. The most potent PEs in this regard were coumestrol (CM), zearalenone (ZR), and genistein (GN). The specificity of PE-induced suppression of basal FSH was indicated by the absence of suppression in cells coincubated with PEs and an estrogen receptor (ER) blocker (ICI 182 780; ICI). Secretion of LH during stimulation by a GnRH agonist (GnRH-A) was used as a measure of gonadotrope responsiveness. Incubation of cells for 12 h with E_2 , CM, ZR, GN, or daidzein (DZ) enhanced the magnitude and sensitivity of LH secretion during subsequent exposure to graded levels of a GnRH-A. The E_2 - and PE-dependent augmentation of gonadotrope responsiveness was nearly fully blocked during coincubation with ICI. Collectively, these data demonstrate that selected PEs (CM, ZR, and GN), like E_2 , decrease basal secretion of FSH, reduce total FSH production, and enhance GnRH-A-induced LH secretion in a manner that is dependent on the ER.

Key Words

- ▶ LH
- ▶ FSH
- ▶ cell culture
- ▶ phytoestrogens
- ▶ estradiol
- ▶ estrogen receptor
- ▶ ovine pituitary cells

Journal of Endocrinology
(2013) 219, 243–250

Introduction

Phytoestrogens (PEs) are nonsteroidal, organic compounds that are bound by one or both of the estrogen receptor (ER) isoforms, ER α and ER β (Kuiper *et al.* 1998). Although nonsteroidal, the structure of these plant-derived compounds generally includes a phenolic ring resembling the aromatic ring of ovarian-derived estrogens, such as 17 β -estradiol (E_2).

Endogenous estrogens affect a diverse array of tissues including the gonadotrope cells of the pituitary. These cells synthesize the gonadotropic hormones, luteinizing

hormone (LH), and follicle-stimulating hormone (FSH), which have important roles in reproduction. Specifically, in females FSH stimulates follicular growth and maturation, whereas LH promotes steroidogenesis and ovulation. Although estrogens can affect gonadotropin synthesis and secretion by influencing gonadotropin-releasing hormone (GnRH) release from hypothalamic loci, direct action of estrogens on gonadotrope cells is suggested by experiments using *in vivo* and *in vitro* models in which pituitary tissue is physically separated from hypothalamic inputs

(Clarke & Cummins 1984). Indeed, effects of estrogens on basal and GnRH-stimulated gonadotropin secretion are evident in cell culture using ovine or rodent pituitary cells (Huang & Miller 1980). Although the effect of endogenous estrogens on gonadotrope function is well recognized, much less is known regarding the effect of PEs on the synthesis and secretion of FSH. This may be a particularly important concern in animal production systems, because several common forages, including alfalfa, clover, lupin, and some grains, contain high concentrations of PEs (Dixon 2004). In addition, the rumen and intestinal microbiota of domestic livestock species may enhance the estrogenic potency of feedstuffs by transforming plant precursors into active PEs (Zhou *et al.* 2009).

The potential effects of PEs on the reproductive efficiency of livestock is suggested by reports that female sheep and cattle grazing on subterranean clover (*Trifolium subterranean*) or alfalfa (*Medicago sativa*) became infertile (Bennetts *et al.* 1946, Moule *et al.* 1963). These effects of PEs on reproduction are likely to reflect PE-dependent change in the ER function. Indeed, Mathieson & Kutts (1980) report that genistein (GN) and coumestrol (CM) compete with E₂ for binding sites in hypothalamic and pituitary tissue of sheep.

In the studies reported here, we examine the physiological effect of PEs commonly present in feedstuffs provided to domestic animals. Our particular objective was to determine the effect of PEs on basal secretion of FSH. In addition, we assessed the effect of PEs on GnRH-induced LH secretion from ovine pituitary cells.

Materials and methods

Reagents and supplies

Calcium and magnesium-free (CMF) Hanks balanced salt solution (HBSS), and medium 199 (without phenol red, L-glutamine, or NaHCO₃; M199) were obtained from Sigma–Aldrich. Bovine insulin, the GnRH agonist (D-Ala⁶, des-Gly¹⁰ GnRH ethylamide; GnRH-A), pancreatin, the ER antagonist (ICI 780 182; fulvestrant), and most PEs were also purchased from Sigma–Aldrich. The PE, daidzein (DZ), was purchased from Tocris Bioscience (Ellisville, MO, USA). The antifungal drug, fungizone, and the two antibiotics, gentamycin sulfate and penicillin/streptomycin, were obtained from HyClone (Logan, UT, USA), Mediatech (Manassas, VA, USA), and Gibco respectively. Collagenase (type-I; Worthington Biochemical Corporation, Lakewood, NJ, USA), L-glutamine (Invitrogen), BSA (fraction V; EMD Chemicals, Gibbstown, NJ, USA),

and E₂ (United States Biochemical Corp., Cleveland, OH, USA) were obtained from established vendors.

Cell culture

Pituitary tissue was collected from castrated male yearling sheep euthanized at a local abattoir by electrical stunning and exsanguination. Tissue was collected within 10 min of euthanasia and immersed in low calcium, magnesium-free (LCMF) HBSS. LCMF HBSS was prepared by supplementing CMF HBSS with CaCl₂·H₂O, 1% BSA, 25 mM HEPES, 0.035% NaHCO₃, and penicillin/streptomycin (100 units/ml). The tissue was dissociated into component cells using a modification of the procedure developed by Huang & Miller (1980). Briefly, anterior pituitary tissue was dissected free of connective tissue and the posterior pituitary and sliced into 0.5 mm sections using a Stadie–Riggs tissue slicer. The pituitary slices were minced into roughly 2 mm² with scissors. The minced tissue from five pituitary glands was combined and washed five times with LCMF HBSS before resuspension into 66 ml LCMF HBSS containing 200 mg collagenase (290 U/mg). The tissue suspension was gently agitated in a rotary shaker (90 r.p.m.) at 37 °C for 90 min. After incubation with collagenase, the partially digested tissue was pelleted by centrifugation (400 g for 4 min). The collagenase containing supernatant was decanted and tissue fragments were resuspended in 60 ml CMF HBSS containing 0.25% pancreatin. Tissue fragments were incubated with pancreatin for 35 min at 37 °C with gentle agitation. At the conclusion of enzyme treatment, undigested tissue was removed by passage of the cell suspension through a nylon mesh filter. Dispersed cells were pelleted by centrifugation (400 g for 4 min) and washed four times with CMF HBSS. Cell yield was assessed using a hemocytometer and viability was determined by dye exclusion. Average yield was 200 × 10⁶ cells/g initial tissue (n = 5), with a viability > 90% (mean = 95.5%). After the final wash with CMF HBSS, the cell pellet was suspended using M199 containing NaHCO₃ (2.2 g/l), L-glutamine (200 mM), insulin (5 µg/ml), gentamycin sulfate (60 µg/ml), penicillin/streptomycin (100 units/ml), fungizone (500 ng/ml), HEPES sodium salt (25 mM), and 10% serum collected from castrated male sheep (M199 + 10% wether serum (WS)). One milliliter aliquot of the cell suspension (0.5 × 10⁶ cells/ml) was used to seed the 24-well culture plates. The seeded plates were placed in a water-jacketed incubator and cells were allowed to attach during 36–48 h culture at 37 °C in a humidified atmosphere of 5% CO₂ and air.

Experimental procedure

The effect of E_2 and PEs on the basal secretion of FSH from attached pituitary cells was assessed by removing conditioned culture media, washing each well with 1 ml M199+10% WS and, finally adding 1 ml M199+10% WS the appropriate concentration of test compound (E_2 with PE). The PEs (CM, zearalenone (ZR), GN, DZ, resveratrol (RV), biochanin A (BA), and enterolactone (EL)) were diluted in DMSO to working concentrations (0.001–1.0 μ M) using M199+10% WS (final DMSO concentration was 0.2%). Vehicle also contained DMSO. We found no evidence that DMSO at the concentration (0.2%) used in these studies influenced the results. Conditioned media was collected after 48 h incubation with vehicle, E_2 , or PE. In some experiments vehicle, E_2 , or PEs were coincubated with an ER antagonist (ICI 182 780). After removal of conditioned media, cellular content of FSH was assessed by adding 1 ml of cold 0.01 M PBS to each well and rapidly freezing plates at -85°C . The attached cells were lysed by repeated freezing and thawing as described in previous studies (Adams *et al.* 1979). PBS was collected after the fourth freeze–thaw cycle and used to determine cellular FSH content. The concentration was then combined with basal secretion to determine the total production of FSH.

The effect of E_2 and PEs on the sensitivity and magnitude of GnRH-A-induced LH secretion was assessed using a related experimental paradigm. Briefly, after a 48 h attachment period, conditioned media was removed and replaced with 1 ml media containing the desired concentration of E_2 , PE, and/or ICI. After 12 h incubation with test compounds, wells were flushed twice with 1 ml media, with final addition of 1 ml media containing vehicle or the appropriate concentration of GnRH-A (0.1–1000 pM). Media from treated wells was collected after a 6 h incubation with the GnRH-A.

Measurement of LH and FSH

The concentrations of LH and FSH were determined by RIA following procedures described previously (Adams *et al.* 1975, 1988). The ovine FSH (NIADDK-oFSH-20) and LH (NIADDK-oLH-26) standard preparations were kindly provided by NIH.

Statistical analysis

The statistical significance of experimental observations was assessed using the mixed model procedure lmer() in R (Bates & Maechler 2009). For studies of basal FSH secretion, fixed

effects included treatment, experiment, and treatment–experiment interaction, while plate was included as a random effect. In contrast, GnRH-A-induced LH secretion was analyzed using a model that included the fixed effects of treatment, GnRH, and treatment–GnRH-A interaction; experiment and experiment–GnRH-A, while plate and plate–GnRH-A were included as random effects. The likelihood-ratio-test was used to determine the significance of the fixed effects ($P < 0.05$) and competing models were fitted using maximum likelihood. Contrasts were setup to determine the mean comparisons against the negative control (vehicle) and positive controls (0.05 and 5 nM E_2). The Bonferroni correction was used to control the type 2 error rate.

Results

Effect of E_2 and PEs on basal gonadotropin parameters

To determine the effects of E_2 and PEs on basal secretion of FSH and total FSH production, we incubated ovine pituitary cells with increasing concentrations of E_2 and PEs for 48 h. Physiologic (0.05 nM) and pharmacologic (5 nM) concentrations of E_2 markedly reduced basal secretion of FSH and significantly reduced total FSH production (Fig. 1). Some, but not all, PEs had a similar effect. The two most potent PEs in this regard were CM and ZR. Neither DZ, RV, BA, nor EL affected basal FSH secretion or total production of FSH in this ovine pituitary cells culture system.

Role of ER on basal FSH parameters

To assess the role of estrogen receptors in expression of the effects of E_2 and PEs, we coincubated pituitary cells with E_2 or PEs and ICI 182 780 (ICI), a potent estrogen receptor antagonist. The results of these coincubation studies clearly demonstrate that estrogen-dependent suppression of both FSH parameters is mediated by ER (Fig. 2). Indeed, the normal estrogen-induced reduction in FSH secretion was blocked, or markedly attenuated in cells receiving the estrogenic stimulus in combination with ICI. It is important to note that ICI not only did block the response induced by E_2 but also prevented the PE-induced decrease in basal FSH secretion (Fig. 2A). Similarly, estrogen-dependent decrease in total FSH production was prevented in cells receiving estrogen and ICI concurrently (Fig. 2B).

Effect of E_2 and PEs on gonadotrope responsiveness

In another set of studies, we examined the effect of E_2 and PEs on the magnitude and sensitivity of GnRH-A-induced

Figure 1

Effect of estrogenic stimuli on (A) basal FSH secretion and (B) total FSH production of ovine pituitary cells treated with various concentrations of estradiol (E_2), coumestrol (■), zearalenone (○), genistein (▲), daidzein (□), resveratrol (●), biochanin A (△), or enterolactone (◆) for 48 h. Control wells were treated with vehicle alone and yielded 49.9 ± 1.2 and 61.6 ± 0.7 ng/ml for basal secretion and total production respectively.

LH secretion. As described in detail in the Materials and methods section, attached cells were pretreated with E_2 , PE, and/or ICI for 12 h and then exposed to vehicle or various concentrations of GnRH-A for 6 h. The magnitude and sensitivity of GnRH-A-induced LH secretion during the 6 h treatment period were determined. The effect of E_2 , ICI, and selected PEs on gonadotrope responsiveness is illustrated in Fig. 3. These data clearly demonstrate that pretreatment with physiological concentration of E_2 significantly enhanced the magnitude and sensitivity of LH secretion induced by GnRH-A. Pretreatment of pituitary cells with selected PEs, including CM, ZR, GN, or DZ, resulted in similar augmentation of gonadotrope

responsiveness. The enhanced gonadotrope responsiveness resulting from pretreatment with E_2 or PEs was markedly attenuated in cells coincubated with estrogenic stimuli and ICI. Interestingly, pretreatment with ICI alone resulted in a modest, but consistent increase in both maximal GnRH-A dependent LH secretion and gonadotrope sensitivity.

The data presented in Table 1 demonstrate that 12 h pretreatment of pituitary cells with E_2 or PEs did not affect subsequent basal secretion of LH. Conversely, the magnitude of LH secretion induced by 1000 pM GnRH-A was significantly increased by prior exposure to physiological

Figure 2

Effect of the estrogen receptor antagonist ICI 182 780 (ICI; 1 μ M) on the decrease in (A) basal secretion of FSH and (B) total FSH production induced by estradiol (E_2), coumestrol (CM), zearalenone (ZR), or genistein (GN). Anterior pituitary cells were treated with vehicle, E_2 (0.05 or 5.0 nM), or 1 μ M concentration of the PEs for 48 h either alone or in combination with 1 μ M ICI. Control wells were treated with vehicle alone and yielded 49.9 ± 1.2 and 61.6 ± 0.7 ng/ml for basal secretion and total production respectively. ***, $P < 0.001$ compared to Vehicle w/ and w/o ICI.

Figure 3

Effect of estradiol (E_2), ICI 182 780 (ICI), and selected PEs (A) coumestrol (CM), (B) zearalenone (ZN), (C) genistein (GN), and (D) daidzein (DZ) on the magnitude and sensitivity of GnRH agonist-induced LH secretion from ovine pituitary cells. Data for vehicle, E_2 , and ICI are identical and were included as a reference for the treatments.

concentration of E_2 (50 pM). Pretreatment with PEs similarly increased the magnitude of GnRH-A-induced LH secretion. The PEs with the highest potency in this regard were CM and ZR, both of which induced the greatest enhancement of secretory response at a PE concentration of 0.1 μ M. The augmented response induced by GN or DZ was somewhat less robust in that 1 μ M concentration of the PE was required to elicit significant enhancement of gonadotrope responsiveness.

In addition to affecting the magnitude of LH secretion induced by high levels of GnRH or GnRH-A stimulation, prior exposure to E_2 or PEs may also affect the sensitivity of the response. We quantified gonadotrope sensitivity by determining the concentration of GnRH-A required to establish the half maximal secretory response. The results of these analyses are presented in Table 2. These data demonstrate that the concentration of GnRH-A required to induce half maximal LH secretion from control cells pretreated with vehicle alone was 35.5 ± 2.8 pM. Pretreatment of pituitary cells with physiological concentrations of E_2 (50 pM) markedly enhanced gonadotrope sensitivity and, as a consequence, lowered the GnRH-A stimulus required to establish the half maximal response to 1.3 ± 2.8 pM. Pretreatment with selected PEs had a similar effect on gonadotrope sensitivity, with CM and ZR being

Table 1 Effect of estradiol (E_2) and increasing concentrations of coumestrol (CM) or zearalenone (ZR) on basal and GnRH-agonist (GnRH-A)-induced LH secretion. After a 48 h period of attachment, pituitary cells were incubated for 12 h with vehicle, E_2 , or increasing concentrations of CM or ZR. The magnitude of LH secretion induced by 1 nM GnRH-A was assessed during the 6 h period that followed estrogen pretreatment. Values represent the mean $\pm$ S.E.M. of at least three replicates of the experiment

Pretreatment	Basal LH (ng/well)	1 nM GnRH-A-induced LH secretion (ng/well)
Vehicle	14.6 $\pm$ 0.5*	45.9 $\pm$ 1.9*
50 pM E_2	14.6 $\pm$ 0.7*	90.2 $\pm$ 5.5 [†]
Coumestrol (nM)		
1	11.6 $\pm$ 0.6*	42.4 $\pm$ 1.0*
10	13.2 $\pm$ 0.8*	67.9 $\pm$ 1.4 [†]
100	12.1 $\pm$ 0.5*	83.1 $\pm$ 3.7 [†]
1000	12.1 $\pm$ 0.8*	78.7 $\pm$ 4.1 [†]
Zearalenone (nM)		
1	11.5 $\pm$ 0.7*	35.8 $\pm$ 1.0*
10	13.5 $\pm$ 0.3*	63.4 $\pm$ 4.1 [†]
100	11.3 $\pm$ 0.8*	77.3 $\pm$ 1.2 [†]
1000	12.1 $\pm$ 0.4*	75.6 $\pm$ 4.1 [†]

*,[†]Values within a column with differing superscripts differed significantly ($P < 0.05$).

Table 2 Effect of estradiol (E₂), selected phytoestrogens (PEs), and/or ICI 182 780 (ICI), an estrogen receptor antagonist, on the sensitivity of GnRH agonist (GnRH-A)-induced LH secretion. The concentration of GnRH-A that effected half maximal LH secretion was used as measure of gonadotrope sensitivity. After a 48 h period of attachment pituitary cells were incubated for 12 h with vehicle, E₂ (50 pM), coumestrol (CM; 0.1 µM), zearalenone (ZR; 0.1 µM), genistein (GN; 1 µM), or daidzein (DZ; 1 µM DZ) alone or in combination with 1 µM ICI. The magnitude of GnRH-A induced LH secretion was assessed during the 6 h period that followed estrogen pretreatment. Values represent the mean $\pm$ s.e.m. of at least three replicates of the experiment

Pretreatment		GnRH-A (pM) ^a
Estrogen	ICI 182 780 (1 µM)	
Vehicle	Vehicle	35.5 $\pm$ 2.8*
Vehicle	ICI	19.1 $\pm$ 2.8 [†]
E ₂ (50 pM)	Vehicle	1.3 $\pm$ 2.8 [†]
E ₂ (50 pM)	ICI	23.7 $\pm$ 2.8 [†]
CM (0.1 µM)	Vehicle	1.2 $\pm$ 2.8 [†]
CM (0.1 µM)	ICI	20.0 $\pm$ 2.8 [†]
ZR (0.1 µM)	Vehicle	1.3 $\pm$ 2.8 [†]
ZR (0.1 µM)	ICI	16.7 $\pm$ 2.8 [†]
GN (1 µM)	Vehicle	0.9 $\pm$ 2.8 [†]
GN (1 µM)	ICI	10.3 $\pm$ 2.8 [§]
DZ (1 µM)	Vehicle	1.4 $\pm$ 2.8 [†]
DZ (1 µM)	ICI	20.0 $\pm$ 2.8 [†]

*,[†],[§]Values with differing superscripts differed significantly ($P < 0.05$).

^aConcentration of GnRH-A required to induce half maximal LH secretion.

most effective in this regard. It is again important to note that the augmentation of gonadotrope sensitivity induced by E₂ and PEs was partially reversed in cells exposed to estrogen together with ICI during the pretreatment period. The other PEs examined, RV, BA, and EL, did not affect either gonadotrope sensitivity or maximal agonist-induced response, even at maximum concentrations (1 µM).

Discussion

The results presented here demonstrate that PEs exert estrogen-like effects at pituitary loci. We demonstrated that PEs, like E₂ itself, suppressed basal FSH secretion and also reduce total FSH production. Similarly, we demonstrated that PE treatments exhibited estrogen-like activity and increased the secretory response induced by GnRH-A, also in a dose-dependent manner. Furthermore, we combined estrogenic stimuli with the 'pure' ER antagonist, ICI, to illustrate that the PE-mediated effects on basal FSH secretion and GnRH-A induced LH secretion are ER-dependent. Interestingly, ICI displayed estrogenic and

anti-estrogenic effects on gonadotrope function in the ovine pituitary cell culture system used in our study.

Estrogenic stimuli are known to attenuate basal FSH secretion and residual intracellular FSH and thereby decrease total FSH production. This has been demonstrated in several species using both *in vivo* and *in vitro* models (Lindzey *et al.* 2006, Arreguin-Arevalo *et al.* 2007). Groups using ovine pituitary cells in culture have clearly demonstrated that treatment with E₂ attenuates FSH secretion and intracellular synthesis (Miller *et al.* 1977, Huang & Miller 1980, Nett *et al.* 2002). In particular, E₂ suppresses these parameters by decreasing FSH β expression (Miller & Miller 1996, Baratta *et al.* 2001). Unfortunately, the specific mechanism by which E₂ exerts this effect is not clear. However, Baratta *et al.* (2001) demonstrated that E₂ decreased activin β , a growth factor necessary for normal FSH β transcription. They concluded that E₂ indirectly regulates FSH β transcription by decreasing activin β and, ultimately, decreasing synthesis and secretion of FSH. Our results are consistent with this model.

It is well established that estrogenic stimuli increase gonadotrope responsiveness. Elevated E₂ concentrations immediately preceding ovulation increase GnRH receptor (GnRHR) expression in gonadotrope cells in sheep (Sakurai & Adams 1991, Turzillo *et al.* 1998, Clarke 2002). Hypothalamic-derived GnRH binds the GnRHR, thereby activating the necessary second messenger cascades, which ultimately increase intracellular calcium concentrations necessary to facilitate LH secretion (Anderson 1996). Our study demonstrates that certain PEs, particularly CM, ZR, GN, and DZ, induce estrogen-like effects on gonadotrope responsiveness. One explanation is that PEs increase GnRHR mRNA and expression in the pituitary like E₂. That this response to E₂ or the PEs requires one or both of the ER isoforms is suggested by our observation that the responses are attenuated by coinubation with the ER antagonist ICI.

The ER antagonist is commonly used *in vivo* and *in vitro* to determine the role of the ER in physiological processes. Upon binding the ER, ICI prevents the receptor dimerization necessary for gene expression (Dauvois *et al.* 1993, Osborne *et al.* 2004). Furthermore, ICI effectively inactivates the ER by blocking the AF1 and AF2 transcription activation domains and ultimately increases ER degradation (Osborne *et al.* 1995, Wakeling 1995). Collectively, these effects of ICI block the effective action of residual ERs. We observed that ICI blocked the effects of E₂ and maximum concentrations of CM, ZR, and GN. Our data illustrate that ICI combined with estrogenic stimuli blocked the suppression of basal FSH secretion and total

production of FSH. This suggests that ICI prevents estrogenic stimuli from attenuating activin β transcription.

Interestingly, ICI exhibited biphasic responses on gonadotrope responsiveness. When estrogenic stimuli were combined with ICI, we recorded a decrease in responsiveness. This observation indicates that E₂ and the PEs exert their effect by interacting with one or both of the ER isoforms in gonadotrope cells. In contrast, ICI alone induced a modest, but significant and repeatable, enhancement of gonadotrope responsiveness and suggests that ICI functions as a partial agonist in our *in vitro* pituitary cell culture system. Although most studies indicate that ICI functions as a pure ER antagonist, recent observations demonstrate that ICI may have partial agonist activity at some tissues. For example, research conducted using rat primary hippocampal neurons also demonstrated that ICI exhibits agonistic activity (Zhao *et al.* 2006).

It is important to note that our *in vitro* cell culture system comprises a heterogeneous population of pituitary cells. The gonadotrope cells predominately express ER α and, therefore, are likely to respond directly to estrogenic inputs (Tobin *et al.* 2001, Clarke 2002). However, many other pituitary cells, including somatotropes and lactotropes, also express ERs and also have the capacity for direct response to estrogens (Tobin *et al.* 2001, Zárate & Seilicovich 2010) or antiestrogens like ICI. This raises the possibility that paracrine signals from adjacent pituitary cells may impinge on the gonadotropes to modulate various aspects cell of function. In this mixed cell system, the measurable parameters, like gonadotrope responsiveness and basal secretion of LH and FSH, may represent the cumulative effect of direct action of estrogens and antiestrogens on gonadotrope cells and indirect estrogenic responses mediated by estrogen-controlled paracrine factors produced by other pituitary cells.

In conclusion, our study illustrates that PEs decrease basal FSH secretion and total FSH production in a manner that is comparable to E₂. This action is blocked when E₂ or PE are combined with the ER antagonist, ICI. Our study also shows that PEs increase gonadotrope responsiveness to the GnRH-A in a manner that is similar to E₂, and this effect is partially reversed by ICI. Our continuing studies are examining the relative important of specific ER isoforms in the expression of the multifaceted effects of estrogens on gonadotrope function.

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 research was supported by a HATCH Project grant (RR-4505) from the USDA.

References

- Adams TE, Kinder JE, Chakraborty PK, Estergreen VL & Reeves JJ 1975 Ewe luteal function influenced by pulsatile administration of synthetic LHRH/FSHRH. *Endocrinology* **97** 1460–1467. (doi:10.1210/endo-97-6-1460)
- Adams TE, Wagner TO, Sawyer HR & Nett TM 1979 GnRH interaction with anterior pituitary. II. Cyclic AMP as an intracellular mediator in the GnRH activated gonadotroph. *Biology of Reproduction* **21** 735–747. (doi:10.1095/biolreprod21.3.735)
- Adams TE, Quirke JF, Hanrahan JP, Adams BM & Watson JG 1988 Gonadotropin-secretion during the periovulatory period in Galway and Finnish Landrace ewes, and Finnish Landrace ewes selected for high ovulation rate. *Journal of Reproduction and Fertility* **83** 575–584. (doi:10.1530/jrf.0.0830575)
- Anderson L 1996 Intracellular mechanisms triggering gonadotrophin secretion. *Reviews of Reproduction* **1** 193–202. (doi:10.1530/ror.0.0010193)
- Arreguin-Arevalo JA, Davis TL & Nett TM 2007 Differential modulation of gonadotropin secretion by selective estrogen receptor 1 and estrogen receptor 2 agonists in ovariectomized ewes. *Biology of Reproduction* **77** 320–328. (doi:10.1095/biolreprod.107.060046)
- Baratta M, West LA, Turzillo AM & Nett TM 2001 Activin modulates differential effects of estradiol on synthesis and secretion of follicle-stimulating hormone in ovine pituitary cells. *Biology of Reproduction* **64** 714–719. (doi:10.1095/biolreprod64.2.714)
- Bates D & Maechler M 2009 lme4: linear mixed-effects models using S4 classes. R package version 0.999375-32.
- Bennetts HW, Underwood EJ & Shier FL 1946 A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Australian Veterinary Journal* **22** 2–12. (doi:10.1111/j.1751-0813.1946.tb15473.x)
- Clarke IJ 2002 Multifarious effects of estrogen on the pituitary gonadotrope with special emphasis on studies in the ovine species. *Archives of Physiology and Biochemistry* **110** 62–73. (doi:10.1076/apab.110.1.62.898)
- Clarke IJ & Cummins JT 1984 Direct pituitary effects of estrogen and progesterone on gonadotropin secretion in the ovariectomized ewe. *Neuroendocrinology* **39** 267–274. (doi:10.1159/000123990)
- Dauvois S, White R & Parker MG 1993 The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *Journal of Cell Science* **106** 1377–1388.
- Dixon RA 2004 Phytoestrogens. *Annual Review of Plant Biology* **55** 225–261. (doi:10.1146/annurev.arplant.55.031903.141729)
- Huang ES & Miller WL 1980 Effects of estradiol-17 β on basal and luteinizing hormone releasing hormone-induced secretion of luteinizing hormone and follicle stimulating hormone by ovine pituitary cell culture. *Biology of Reproduction* **23** 124–134. (doi:10.1095/biolreprod23.1.124)
- Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B & Gustafsson J-A 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor- β . *Endocrinology* **139** 4252–4263. (doi:10.1210/en.139.10.4252)
- Lindzey J, Jayes FL, Yates MM, Couse JF & Korach KS 2006 The bi-modal effects of estradiol on gonadotropin synthesis and secretion in female mice are dependent on estrogen receptor- α . *Journal of Endocrinology* **191** 309–317. (doi:10.1677/joe.1.06965)
- Mathieson RA & Kutts WD 1980 Binding of phyto-oestrogen and oestradiol-17 β by cytoplasmic receptors in the pituitary gland and hypothalamus of the ewe. *Journal of Endocrinology* **85** 317–325. (doi:10.1677/joe.0.0850317)

- Miller CD & Miller WL 1996 Transcriptional repression of the ovine follicle-stimulating hormone- β gene by 17 β -estradiol. *Endocrinology* **137** 3437–3446. (doi:10.1210/en.137.8.3437)
- Miller WL, Knight MM, Grimek HJ & Gorski J 1977 Estrogen regulation of follicle-stimulating hormone in cell-cultures of sheep pituitaries. *Endocrinology* **100** 1306–1316. (doi:10.1210/endo-100-5-1306)
- Moule GR, Braden AWH & Lamond DR 1963 The significance of oestrogens in pasture plants in relation to animal production. *Animal Breeding Abstracts* **31** 139–157.
- Nett TM, Turzillo AM, Baratta M & Rispoli LA 2002 Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. *Domestic Animal Endocrinology* **23** 33–42. (doi:10.1016/S0739-7240(02)00143-1)
- Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, McCue BL, Wakeling AE, McClelland RA, Manning DL & Nicholson RI 1995 Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. *Journal of the National Cancer Institute* **87** 746–750. (doi:10.1093/jnci/87.10.746)
- Osborne CK, Wakeling A & Nicholson RI 2004 Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *British Journal of Cancer* **90** S2–S6. (doi:10.1038/sj.bjc.6601629)
- Sakurai H & Adams TE 1991 Gonadotrope responsiveness in orchidectomized sheep: I. Effect of continuous infusion of estradiol. *Biology of Reproduction* **45** 804–810. (doi:10.1095/biolreprod45.6.804)
- Tobin VA, Pompolo S & Clarke IJ 2001 The percentage of pituitary gonadotropes with immunoreactive oestradiol receptors increases in the follicular phase of the ovine oestrous cycle. *Journal of Neuroendocrinology* **13** 846–854. (doi:10.1046/j.1365-2826.2001.00701.x)
- Turzillo AM, Nolan TE & Nett TM 1998 Regulation of gonadotropin-releasing hormone (GnRH) receptor gene expression in sheep: interaction of GnRH and estradiol. *Endocrinology* **139** 4890–4894. (doi:10.1210/en.139.12.4890)
- Wakeling AE 1995 Use of pure antioestrogens to elucidate the mode of action of oestrogens. *Biochemical Pharmacology* **49** 1545–1549. (doi:10.1016/0006-2952(94)00528-T)
- Zárate S & Seilicovich A 2010 Estrogen receptors and signaling pathways in lactotropes and somatotropes. *Neuroendocrinology* **92** 215–223. (doi:10.1159/000321683)
- Zhao L, O'Neill K & Brinton RD 2006 Estrogenic agonist activity of ICI 182 780 (Faslodex) in hippocampal neurons: implications for basic science understanding of estrogen signaling and development of estrogen modulators with a dual therapeutic profile. *Journal of Pharmacology and Experimental Therapeutics* **319** 1124–1132. (doi:10.1124/jpet.106.109504)
- Zhou W, Wang G, Han Z, Yao W & Zhu W 2009 Metabolism of flaxseed lignans in the rumen and its impact on ruminal metabolism and flora. *Animal Feed Science and Technology* **150** 18–26. (doi:10.1016/j.anifeedsci.2008.07.006)

Received in final form 16 September 2013

Accepted 18 September 2013

Accepted Preprint published online 19 September 2013