The bi-modal effects of estradiol on gonadotropin synthesis and secretion in female mice are dependent on estrogen receptor-α

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Abstract

Depending on the estrous/menstrual cycle stage in females, ovarian-derived estradiol (E2) exerts either a negative or a positive effect on the hypothalamic–pituitary axis to regulate the synthesis and secretion of pituitary gonadotropins, LH, and FSH. To study the role of estrogen receptor-α (ERα) mediating these effects, we assessed the relevant parameters in adult wild-type (WT) and ERα-null (ERKO) female mice in vivo and in primary pituitary cell cultures. The ERKO mice exhibited significantly higher plasma and pituitary LH levels relative to WT females despite possessing markedly high levels of circulating E2. In contrast, hypothalamic GnRH content and circulating FSH levels were comparable between genotypes. Ovariectomy led to increased plasma LH in WT females but no further increase in ERKO females, while plasma FSH levels increased in both genotypes. E2 treatment suppressed the high plasma LH and pituitary Lhb mRNA expression in ovariectomized WT females but had no effect in ERKO. In contrast, E2 treatments only partially suppressed plasma FSH in ovariectomized WT females, but this too was lacking in ERKO females. Therefore, negative feedback on FSH is partially E2/ERα mediated but more dependent on ovarian-derived inhibin, which was increased threefold above normal in ERKO females. Together, these data indicate that E2-mediated negative feedback is dependent on functional ERα and acts to primarily regulate LH synthesis and secretion. Studies in primary cultures of pituitary cells from WT females revealed that E2 did not suppress basal or GnRH-induced LH secretion but instead enhanced the latter response, indicating that the positive influence of E2 on gonadotropin secretion may occur at the level of the pituitary. Once again this effect was lacking in ERKO gonadotropes in culture. These data indicate that the aspects of negative and positive effects of E2 on gonadotropin secretion are ERα dependent and occur at the level of the hypothalamus and pituitary respectively.


Introduction

Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile pattern from the hypothalamus into the hypothalamic–hypophyseal portal veins that drain into the anterior pituitary, and is the primary stimulus for the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the latter organ (Gharib et al. 1990, Haisenleder et al. 1994, Vale et al. 1994, Shupnik 1996). However, the endocrine role of gonad–derived steroids and peptides (e.g. estradiol (E2) and inhibins) on the hypothalamic–pituitary (HP) axis to modulate gonadotropin synthesis and secretion is also well described and critical to reproductive function (Gharib et al. 1990, Haisenleder et al. 1994, Vale et al. 1994, Shupnik 1996). In most mammals, the estrous cycle is dictated by the bi-modal actions of ovarian-derived estrogens on the HP axis such that moderate levels of E2 during early folliculogenesis are suppressive to LH secretion, while an acute rise in E2 levels at proestrus acts to prime the hypothalamus and/or pituitary to produce the hallmark pre-ovulatory gonadotropin surge that induces ovulation (Freeman 1994). The mechanisms and precise sites of action by which estrogens exert both negative and positive effects on the HP axis are still under investigation. Feedback at the level of the hypothalamus is illustrated by reports that ovariectomy results in upregulation of GnRH and that exogenous E2 treatment of ovariectomized female rodents restores to normal the hypothalamic levels of GnRH mRNA (Zoeller & Young 1988, Zoeller et al. 1988), GnRH content (Wise et al. 1981a), and GnRH secretion (Wise & Ratner 1980, Wise et al. 1981b). However, in vitro studies demonstrate that some effects of E2 on the pituitary are clearly independent of hypothalamic influence, including a suppression of Fshb (FSH-β mRNA) expression (Miller & Miller 1996; sheep) and an increase of Lhb (LH-β mRNA) expression (Shupnik et al. 1989a; rat). Furthermore, such effects of E2 on gonadotropin gene expression appear to be estrogen receptor (ER) mediated, either via estrogen–response elements within the promoter regions of the gonadotropin subunit genes (Shupnik et al. 1989b).
or via interactions with other key transcription factors (Miller & Miller 1996). E2 may also directly modulate the pituitary response to GnRH via regulation of GnRH-receptor (GnRH-R) levels (Naik et al. 1984, Turgeon et al. 1996).

Historically, a single form of nuclear ER, now known as ERα, was thought to mediate the effects of E2 on gonadotropin secretion. However, the discovery of a second form of nuclear ER, the ERβ (Kuiper et al. 1996, Mosselman et al. 1996, Tremblay et al. 1997), presents the possibility that two independent receptor forms may mediate the differential effects of E2 feedback on the HP axis. The comparable binding affinities of ERα and ERβ for various estrogenic ligands (Kuiper et al. 1997) have hampered the development of selective agonists or antagonists that could be employed to differentiate the actions of each receptor form. Therefore, the ERα and ERβ-null (αERKO and βERKO respectively) provide unique models to discern the contribution of each receptor form in mediating the feedback effects of E2 in the HP axis. ERα-null female mice have a hypoplastic reproductive tract. They are anovulatory, invariably possess ovaries that exhibit multiple atretic and enlarged cystic follicles and hypertrophied theca (Couse & Korach 1999). Plasma levels of gonadal steroids are significantly elevated in αERKO females compared with wild-type (WT) females (Couse et al. 2003), as are plasma LH concentrations (Couse et al. 2003), presumably due to the lack of E2-mediated negative feedback in the HP axis of αERKO females. However, plasma FSH levels are similar in αERKO and WT adult female mice (Couse et al. 2003).

Both ERα and ERβ transcripts and immunoreactivity have been localized to hypothalamic nuclei thought to be involved in regulating GnRH secretion in rats and mice (Li et al. 1997, Shughrue et al. 1997a, Laflamme et al. 1998). Furthermore, high-affinity E2 binding and transcripts encoding ERβ (Skyrner et al. 1999, Hrabovszky et al. 2000), estrogen-related receptor-α (ERRα; Herbison & Pape 2001) but not ERα (Herbison & Pape 2001) are reportedly present in GnRH-secreting neurons of the mouse hypothalamus; thereby, challenging the long-held hypothesis that estrogen actions in these cells are indirect. In the pituitary, both ERα and ERβ are expressed in the adult rat (Wilson et al. 1998), whereas adult mice may possess ERα only (Couse et al. 1997, Couse & Korach 1999).

Thus, in the present study, we characterized the role of ERα in regulating gonadotropin synthesis and secretion by evaluating and comparing the following parameters in wild-type and αERKO female mice: (1) hypothalamic GnRH content, (2) circulating and pituitary gonadotropin levels, (3) effects of E2 replacement on circulating gonadotropin levels and pituitary Lh expression in ovariectomized wild-type and αERKO females, and (4) secretory response of gonadotropes to E2 and GnRH challenges in vitro. Our results indicate that female αERKO mice exhibit normal hypothalamic GnRH content, increased plasma and pituitary LH levels despite excessively high levels of circulating E2, but relatively normal plasma FSH levels and low pituitary FSH levels that may be attributed to increased ovarian production of inhibin. Furthermore, ovariectomized αERKO females were refractory to the negative feedback effects of exogenous E2 that effectively suppressed LH gonadotropin synthesis and secretion in WT females. Likewise, αERKO pituitary cells in culture were refractory to the positive effects of E2 that effectively increased GnRH-stimulated LH release from WT pituitary cells.

Materials and Methods

Animals

All procedures involving animals were pre-approved by the National Institute of Environmental Health Sciences (NEIHS) Animal Care and Use Committee. Animals were maintained in plastic cages in a temperature-controlled room (21–22 °C) under a 12 h light:12 h darkness schedule and provided with NIH 31 mouse chow and fresh water available ad libitum. WT (Esr1+/+) and Esr1−/− (αERKO) mice of the C57BL/6 strain were obtained from our colony at Taconic Farms, Germantown, NY, USA and used at 10–16 weeks of age. All animals were genotyped by PCR, on DNA extracted from tail biopsies using the Wizard SV 96 Genomic DNA extraction kit (Promega) as previously described (Couse et al. 2003). Mice were ovariectomized using isoﬂurane anesthesia according to NEIHS approved surgical procedures and allowed to rest 2 weeks prior to experimental use.

Experimental design

Experiment 1 Intact, adult WT and αERKO female mice were killed during 0900–1100 h to determine the basal levels of (1) hypothalamic GnRH content, (2) pituitary gonadotropin content, (3) plasma gonadotropin levels, and (4) serum and plasma inhibin-A levels. Due to sample limitations, not all endpoints were measured in the same group of animals. Animals were killed by CO2 asphyxiation, whole blood was immediately collected from the inferior vena cava; hypothalami and pituitaries were then immediately removed and snap-frozen, and then stored at −70 °C until analysis. Whole blood or whole blood mixed with heparin (60 mg/ml) was centrifuged at 8000 g at 4 °C to collect serum or plasma respectively and stored at −70 °C until further analysis.

Experiment 2 To examine the effect of ovariectomy and E2 replacement on plasma gonadotropin levels and pituitary gonadotropin gene expression, ovariectomized adult female WT and αERKO mice were injected subcutaneously with 100 μl vehicle (sesame oil) or 17β-E2 (Research Plus, Inc., Manasquan, NJ, USA) at 50 μg/kg body weight for three consecutive days between 0900 and 1100 h. E2 treatments of 15–50 μg/kg per day for three consecutive days consistently induce a maximum uterotrophic response in ovariectomized
WT mice (Lubahn et al. 1993, Hewitt et al. 2003). We used the higher dose in the present studies to more closely mimic the elevated E2 levels that are endogenous to intact zERKO female mice (Couse et al. 2003). Blood and tissues were collected 24 h after the final treatment.

Experiment 3 To examine the effects of E2 on basal and GnRH-induced LH secretion in the absence of hypothalamic influence, dispersed pituitary cell cultures were prepared from adult WT and zERKO females according to the procedure of Huang et al. (2001) with the following modifications. Pituitaries were harvested and pooled according to genotype \( (n > 9 \) per genotype per experiment), then minced in 1× Hank’s balanced salt solution (HBSS; Invitrogen) supplemented with 25 mM HEPES (Sigma) and 0.15 mM calcium chloride. The resulting tissue fragments were then digested in HBSS with HEPES containing 0.5 mM collagenase (640 U/ml; 200 μl/5 pituitaries; Worthington, Lakewood, NJ, USA) at 36.5°C for 2–3 h with periodic vortexing; followed by incubation in calcium–magnesium free HBSS with HEPES containing 0.25% pancreatin (Invitrogen) for 15–20 min at 36.5°C. The dispersed cells were then vigorously vortexed, pelleted, and washed three times in culture media (DMEM without phenol red; 10% stripped fetal calf serum; with PenStrep; Invitrogen) and filtered through 50 μm Nitex (Sefar Filtration, Depew, NY, USA) to remove aggregates and debris. Cells were counted and plated (1.5x10⁵ cells/well; four to five wells per treatment) in 96-well plates coated with Matrigel (BD Biosciences, San Jose, CA, USA) diluted 1:3 with DMEM. Cells were incubated in a humidified chamber of 95% O2:5% CO2 at 37°C. After 24 h, the media were changed to culture media containing either vehicle (ethanol) or E2 (10 and 100 pg/ml). After 48 h, the spent medium was carefully removed by aspiration and replaced with experimental medium (DMEM without phenol red; no serum; 0.1% BSA) containing vehicle or E2 (10 and 100 pg/ml), and/or GnRH (10 nM; Sigma). The cells were allowed to incubate for an additional 2 h, after which the media were rapidly collected and stored at −70°C until analysis. The cells were then processed later for RNA extraction. This experiment was repeated three times with similar results.

RNA isolation and analysis

Total RNA was isolated from individual snap-frozen pituitaries or pituitary cell cultures using Trizol reagent according to the manufacturer’s protocol. Glycogen (10 μg/tube) was added prior to the final alcohol precipitation to maximize RNA yield. The concentration of all final preparations was calculated via an A260 reading using a Molecular Devices Spectramax (Sunnyvale, CA, USA) spectrophotometer followed by electrophoresis of a 1 μg aliquot to ensure integrity prior to further analysis.

Pituitary levels of Lhb mRNA were assessed by northern blot analysis on 1 μg aliquots of total RNA from individual pituitaries, then normalized by subsequent probing for Rpl7 mRNA as previously described (Lindzey et al. 1998). Pituitary levels of Fshb mRNA were assessed by ribonuclease protection assays (RPAs) on 1 μg aliquots of total RNA from individual pituitaries, and included a probe for Ppia (cyclophilin) to normalize among samples, as previously described (Couse et al. 2003).

Gene expression in total RNA from cultured pituitary cells was assessed by quantitative real-time reverse-transcriptase PCR (qRT-PCR). For each sample (each well), cDNA was generated from 1 μg RNA in a 25 μl reaction mixture using random hexamers and the superscript cDNA synthesis system (Invitrogen) according to the manufacturer’s protocol. Applied
Biosystems Primer Express (Foster City, CA, USA) software was used to select primers specific for the amplification of murine gonadotropin-releasing hormone receptor (Gnrhr), common α-glycoprotein (Cga), Lhb, and Fshb cDNAs (Table 1). All primer sets were designed to lie in separate exons to avoid erroneous amplification of contaminating genomic DNA and confirmed to amplify a single product of the expected size via dissociation analysis and gel electrophoresis. Each sample was assayed in duplicate using the equivalent of 0.1 μl cDNA (prepared as described previously), 20 pmol of each primer and 1X SYBR Green Master Mix (Applied Biosystems) in a total reaction volume of 50 μl. For normalization purposes, an identical set of reactions was prepared using primers specific for Rpl7 as previously described (Hewitt et al. 2003; Table 1). Amplification was carried out in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as follows: 50 °C/2 min, 95 °C/10 min (1X); 95 °C/15 s, 60 °C/30 s (40X). Quantitative differences in the cDNA target between samples were determined using the mathematical model of Pfaffl (2001), in which an expression ratio was determined for each sample by calculating \( \frac{(E_{\text{target}})^{\Delta Ct(\text{target})}}{(E_{\text{Rpl7}})^{\Delta Ct(\text{Rpl7})}} \), where \( E \) is the efficiency of a primer set and \( \Delta Ct = Ct(\text{calibrator sample}) - Ct(\text{experimental sample}) \) and calibrator sample = control = WT noGnRH noE2. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log μl cDNA vs Ct value over at least four orders of magnitude (\( E = 10^{-\frac{1}{(1/slope)}}, \)).

**Statistical analysis**

All data were analyzed using Levene’s test for homogeneity of variance. If significant heteroscedacity was observed, data were log transformed prior to statistical analyses. Data were initially analyzed using a two-way ANOVA and the Bonferroni–Dunn post hoc test. If significant genotype and treatment interactive effects were noted, data for each genotype were analyzed separately using one-way ANOVA and the Bonferroni–Dunn post hoc test or by \( t \)-tests. In addition, some comparisons between intact WT and zERKO females were made using one-tail, unpaired \( t \)-tests. In all cases, statistical significance was accepted at \( P < 0.05 \).

**Results**

**Experiment I: evaluation of reproductive hormone levels in intact WT and zERKO females**

Despite possessing comparable levels of hypothalamic GnRH content (Fig. 1A), zERKO females exhibited conspicuous dysregulation of gonadotropin synthesis and secretion in the pituitary compared with WT females (Fig. 1B and C). Pituitary LH content and plasma LH levels were both increased 2-7- \( (P<0.05) \) and 8-fold \( (P<0.05) \) respectively, in zERKO females relative to wild type (Fig. 1C). In contrast, plasma FSH levels did not differ between genotypes, but the average pituitary FSH content in zERKO females was 50% below that of WT females \( (P<0.05) \). Inhibin-A levels in zERKO females were dramatically increased (3-7-fold; \( P<0.05 \)) compared with WT females (Fig. 1D).

**Experiment II: effect of ovariectomy and E2 treatment on the zERKO HP axis in vivo**

As expected, ovariectomy led to plasma FSH and LH levels that were increased almost 4- and 12-fold respectively in WT females (Figs 1 and 2). Ovariectomized zERKO females exhibited similar increases in plasma FSH but showed no further rise in plasma LH levels compared with intact zERKO females (Figs 1 and 2). In ovariectomized WT females, E2 treatments suppressed plasma LH levels to pre-surgery levels but decreased the heightened plasma FSH levels by only 30% (compare Figs 1 and 2). In contrast, E2 treatments of ovariectomized zERKO females failed to suppress plasma LH and FSH levels.

The effects of ovariectomy and E2 replacement on plasma gonadotropin levels in each respective genotype were mirrored by changes in Lhb expression in the pituitary. As previously reported, intact zERKO females possessed significantly increased levels of Lhb expression in the pituitary (Couse et al. 2003). As shown in Fig. 2, ovariectomy abolished the genotypic difference in Lhb expression. However, while WT females exhibited a 33% \( (P<0.05) \) reduction in Lhb mRNAs following exogenous E2 treatment, zERKO females exhibited no such change.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Amplified sequences (bp)</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
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<td>NM 009889</td>
<td>182–281</td>
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<td>AGAAGCAACAGCCCATACACTG</td>
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<tr>
<td>Fshb</td>
<td>NM 008045</td>
<td>28–153</td>
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<td>CTCAGATGTTGATGTGTTGGTCATT</td>
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<tr>
<td>Gnrhr</td>
<td>NM 010323</td>
<td>767–866</td>
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<td>GAGTACGCAATGCCAATGCTTC</td>
</tr>
<tr>
<td>Lhb</td>
<td>NM 008497</td>
<td>100–204</td>
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<td>GCACTAACGCGACGATCGTCC</td>
</tr>
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<td>416–436</td>
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<td>GACCAAGGAGCTGCAAGACCT</td>
</tr>
</tbody>
</table>

**Table 1 Primers used for RT-PCR**

Experiment III: effect of E\(_2\) treatment on WT and αERKO pituitary cells in vitro

To examine any direct effects that E\(_2\) may have on gonadotrope function, basal and GnRH-induced LH secretions were evaluated in primary pituitary cell cultures from WT and αERKO females. Cells were first exposed to either vehicle or E\(_2\) (10 or 100 pg/ml) for 48 h and then subjected to an acute (2 h) challenge with GnRH in the continued presence or absence of E\(_2\). Interestingly, αERKO pituitary cells exhibited increased basal Lhb expression and LH secretion relative to WT cells following 48 h in culture, regardless of the presence or dose of E\(_2\) (Fig. 3). Upon GnRH stimulation, non-estrogen exposed WT pituitary cells exhibited a 13-fold increase in LH secretion (\(P<0.05\)), and this was further enhanced to >20-fold in WT cells when pretreated with 10 or 100 pg E\(_2\)/ml (\(P<0.05\) versus no E\(_2\); Fig. 3). αERKO pituitary cells also exhibited increased LH secretion (two- to fourfold, depending on E\(_2\) dose) when challenged with GnRH; however, prior E\(_2\) exposure had no enhancing effect (Fig. 3). The GnRH stimulated LH-secretory response from αERKO pituitary cells, when expressed as fold increase over basal LH secretion, was blunted in comparison with the WT response. However, the absolute GnRH-stimulated LH secretory response reached similar levels in αERKO (21.3 ± 0.8 ng/ml) and WT pituitary cells (20.4 ± 0.2 ng/ml). In contrast to the effect of GnRH on LH secretion, no parallel increase in Lhb expression was observed in either genotype (Fig. 3). Similar assays for Cgα, Fshb, and Gnrhr expression indicated no significant genotypic or treatment effects (data not shown).

Discussion

The endocrine actions of ovarian-derived E\(_2\) on the HP axis are vital to gonadal function and fertility in female mammals. The presence of both known ER forms in the hypothalamus and pituitary, however, confounds our abilities to discern the precise site of such actions and the contribution of each receptor form in mediating the estrogenic effects. We contend that this obstacle may be largely overcome by the study of αERKO mice. Since these animals lack functional ER\(\alpha\) but maintain normal ER\(\beta\) expression (Couse et al. 1997), they are especially suited to reveal phenotypes that may distinguish the actions of the two ERs. Herein, we have employed αERKO female mice to demonstrate that ER\(\alpha\) functions are fundamental to the negative-feedback actions of E\(_2\) in the HP axis, congruent with earlier reports (Wersinger et al. 1999, Couse & Korach 1999, Couse et al. 1999, 2003).

Furthermore, our studies indicate that ER\(\alpha\)-mediated actions are critical to the negative modulation of LH secretion, while FSH secretion is only partially mitigated by ER\(\alpha\)-E\(_2\) actions and are more effectively regulated by ovarian-derived inhibin. We have also found evidence that ER\(\alpha\)-mediated actions are important for the positive modulation of LH secretion at the pituitary, where E\(_2\) effectively increased GnRH-stimulated LH release.

The elevated pituitary and plasma LH levels consistently found in αERKO females are demonstrative of the critical role for ER\(\alpha\) in negatively regulating LH synthesis and secretion. WT females exhibited comparable increases in plasma LH only after being rid of circulating sex steroids via

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**Figure 1** Reproductive hormone levels in the plasma of intact wild-type (WT) and αERKO females. Shown are the levels (mean ± S.E.M.) of (A) hypothalamic gonadotropin-releasing hormone (GnRH) content, (B) pituitary (top) and plasma (bottom) follicle-stimulating hormone (FSH) levels, (C) pituitary (top) and plasma (bottom) luteinizing hormone (LH) levels, and (D) circulating inhibin-A levels. Statistically significant differences between genotypes were determined by Student’s t-test (\(P<0.05\)) and are indicated by an asterisk. Sample sizes equaled 7–10 per genotype for the data shown in (A)–(C), and ≥21 per genotype for the data shown in (D).
ovariectomy. Similarly, female mice devoid of endogenous E2 due to targeted disruption of the Cyp19 (P450 aromatase) gene also exhibit increased plasma LH, and this is abated upon exogenous E2 treatments (Britt et al. 2004). aERKO females consistently possess increased LH levels despite possessing excessively high levels of circulating E2 (Couse et al. 2003) and normal ERβ expression (Couse et al. 1997). Furthermore, ERβ-null (βERKO) females exhibit normal plasma LH levels (Couse et al. 2003). These data collectively indicate that E2-mediated negative feedback on the HP axis is an ERα-specific action and that any role of ERβ is minimal.

In contrast to LH, FSH synthesis and secretion were not increased in intact aERKO females. As ovariectomy resulted in large increases in circulating FSH in both genotypes, an ovarian factor(s) is obviously required to maintain normal FSH synthesis and secretion, and this factor is also present in the aERKO. E2 and inhibins are the two primary ovarian-derived hormones that feedback upon the female HP axis to negatively modulate gonadotropin secretion. We have previously reported that aERKO females possess plasma E2 levels that are elevated almost eightfold above those of WT females (320 ± 17 vs 40 ± 3 pg/ml; Couse et al. 2003). Interestingly, E2 treatments only partially suppressed (30%) FSH levels in ovariectomized WT females. Thus, unlike the regulation of LH secretion, E2 may only play a minor role within the HP axis in negatively regulating FSH secretion, and this effect is mediated by ERα and not ERβ, as E2 treatments of aERKO females completely failed to suppress FSH levels. Congruent with the above findings, the regulation of FSH synthesis and secretion is known to be more dependent on the inhibin/activin family of peptide hormones (Woodruff & Mather 1995, Gregory & Kaiser 2004). Therefore, the reason that FSH levels are not elevated in aERKO females is probably due to their plasma inhibin-A levels, which were threefold above normal. Circulating inhibin-A is primarily derived from the granulosa cells of large, pre-ovulatory follicles in the ovary (Rajkovic et al. 2006). Therefore, increased circulating inhibin-A in aERKO females is not unexpected given that these animals are anovulatory and exhibit ovaries that consistently possess multiple differentiated, albeit unhealthy, follicles (Couse & Korach 1999). Additional markers also considered to be indicative of pre-ovulatory follicles, such as E2 synthesis (Couse et al. 2003) and LH receptor (Couse & Korach 1999), are also increased in aERKO ovaries. Interestingly, female CYP19-null mice share several aspects of the aERKO ovarian phenotype but exhibit a greater than eightfold decrease in inhibin-A levels compared with WT females (Britt et al. 2005) and, accordingly, possess severely elevated levels of plasma FSH (Fisher et al. 1998). Therefore, the once perplexing difference in plasma FSH levels between aERKO and CYP19-null mice is due to a drastic disparity in circulating inhibin-A levels. These data suggest that ligand-dependent actions of ERβ in granulosa cells may facilitate inhibin synthesis in the ovary by promoting granulosa cell growth and function.

The precise site of ERα-mediated negative feedback on gonadotropin synthesis and secretion may be inferred by our findings that basal LH secretion by cultured pituitary cells from either genotype was not altered by E2. This absence of direct E2 effects on gonadotrope behavior strongly suggests that E2/ERα-mediated negative feedback occurs at the level of the hypothalamus. Other studies also indicate that E2 negatively modulates gonadotropin secretion by decreasing the frequency of hypothalamic GnRH pulses (Sarkar & Fink 1980, Weick & Noh 1984). The loss of ERα-mediated E2 actions leading to increased frequency of GnRH secretion may also lead to increased GnRH synthesis, resulting in unaltered net hypothalamic GnRH content, congruent with our observation that aERKO females exhibited hypothalamic GnRH content levels that were not different from wild type.

Despite the above findings, ERα-null gonadotropes placed in culture and therefore removed from any hypothalamic influence,

Figure 2 Pituitary gonadotropin gene expression and plasma gonadotropin levels in ovariectomized and estradiol-treated (E2) female mice. Shown are the levels (mean ± S.E.M.) of plasma follicle-stimulating hormone (FSH) (left) and plasma luteinizing hormone (LH) and pituitary Lhb mRNA (right) in ovariectomized wild-type (WT) and aERKO females following treatment with either vehicle (sesame oil) or E2 (50 μg/kg body weight, once per day for 3 days). Lhb mRNA levels were determined by northern blot and normalized to levels of Rpl7 mRNA per sample; sample sizes were seven to eight animals per group. Statistical differences between genotypes and treatments, as indicated by *, were determined by first employing a two-way ANOVA (P < 0.05), followed by the Bonferroni–Dunn post hoc test (P < 0.05 vs vehicle-treated WT) where appropriate.
continue to exhibit increased basal LH secretion and Lhb expression relative to WT gonadotropes. A difference in the gonadotrope population between WT and αERKO pituitaries is an unlikely explanation for the increased in vitro basal LH secretion in the latter. In fact, female αERKO pituitaries do not exhibit an increased number of gonadotropes (Scully et al. 1997) and are slightly decreased in weight (data not shown). Furthermore, our finding that other relevant gonadotrope mRNAs, such as Cga, Fshb, or Gnrhr, were not similarly increased in cultured αERKO pituitary cells supports the existence of a comparable gonadotrope population in αERKO pituitaries. Expression of other regulators known to be involved in gonadotrope function and regulation, such as Nr5a1 (SF-1), Egr-1, or Nr0b1 (DAX-1; Achermann & Jameson 1999) may also be altered and may contribute to the abnormally high LH synthesis and secretion in the αERKO. The preservation of abnormally high LH synthesis and secretion by ERα-null pituitary cells, even when removed from hypothalamic influence, may be due to lingering effects of chronic GnRH hyperstimulation of the αERKO pituitary prior to tissue collection. Indeed, Lhb mRNA levels are reported to remain stable for several days following GnRH blockade in male rats (Paul et al. 1990). Alternatively, abnormal LH secretion and Lhb expression in αERKO gonadotropes could be attributed to aberrant development of the anterior pituitary due to the absence of ERα.

In contrast to the negative effects of E2/ERα actions on LH secretion that occurs primarily via the hypothalamus, our in vitro studies indicated that E2 had a positive effect at the level of the pituitary. E2 enhanced GnRH-induced LH secretion in cultured pituitary cells from WT mice. E2 priming before GnRH stimulation did not lead to similar increases in Cga, Fshb (data not shown), or Lhb expression in cells from either genotype, indicating that this is primarily a secretory response. Gnrhr expression in these cultures was also unaltered (data not shown), but our analyses are limited to the transcriptional levels and may not reflect true Gnrhr-R protein levels or even the level of active receptor on the gonadotrope cell surface.

However, E2 treatment of αERKO-derived pituitary cells failed to enhance LH-secretory responses to GnRH, indicating that this positive effect of E2 on LH secretion is also dependent on the presence of functional ERα. The fact that in our cultures, the absolute amount (ng/ml) of LH released in response to a GnRH stimulus was not greater in αERKO than in WT pituitary cells suggests that the elevated levels of plasma LH consistently present in αERKO females stem from high hypothalamic GnRH secretion and not from increased pituitary responsiveness.

An attractive experimental use of αERKO mice is to explore E2-mediated actions within the HP axis that may be independent of ERα. For example, we have previously reported that Esr2 (ERβ) expression in the hypothalamus and pituitary of αERKO females is not different from wild type (Couse et al. 1997, Couse & Korach 1999), and therefore any role of ERβ in mediating E2 actions is presumably intact. The possible involvement of ERβ allows for the intriguing prospect that the bi-modal feedback effects of E2 on gonadotropin regulation are via a dual-receptor system. However, the present study produced no evidence that ERβ is involved in mediating the positive feedback actions of E2 at the pituitary. Indeed, female βERKO mice are able to spontaneously ovulate and therefore presumably capable of producing a gonadotropin surge at proestrus (Couse & Korach 1999), but they also exhibit reduced fecundity that

![Figure 3](image-url)
may be attributed to infrequent and/or blunted LH surges. ERβ has been postulated to facilitate the LH surge by mediating an estrogen-induced increase in progesterone receptor (PR) expression in the hypothalamus, which is required for the LH surge (Chappell et al. 1997, Chappell & Levine 2000). This is supported by the preservation of E2-induced PR expression in the hypothalami of αERKO females (Shughrue et al. 1997b, Moffatt et al. 1998). In addition, ERα and ERβ transcripts are detected in an immortalized GnRH neuronal cell line (Butler et al. 1999, Roy et al. 1999), while ERβ but not ERα transcripts are reportedly present in GnRH-secreting neurons within the medial preoptic area of female rats (Hrabovszky et al. 2000).

In summary, our data indicate that E2/ERα actions are critical to the negative modulation of LH synthesis and secretion, but less important to the regulation of FSH synthesis and secretion in female mice, and both of these actions occur primarily at the level of the hypothalamus. Conversely, the ability of E2 to enhance the GnRH response of wild type, but not αERKO gonadotropes in culture indicates that the positive influence of E2 on gonadotropin secretion is also ERα mediated, but occurs at the level of the pituitary. Thus, ERα is responsible for aspects of both negative and positive feedback effects of E2 on LH synthesis and secretion.

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