DISSERTATION

A NON-GENOMIC ACTION OF 17 β-ESTRADIOL AS THE MECHANISM UNDERLYING THE ACUTE SUPPRESSION OF SECRETION OF LH IN PRIMARY CULTURES OF OVINE PITUITARY CELLS AND IN OVARIECTOMIZED EWES

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JESUS ALEJANDRO ARREGUIN AREVALO ENTITLED A NON-GENOMIC ACTION OF 17 β-ESTRADIOL AS THE MECHANISM UNDERLYING THE ACUTE SUPPRESSION OF SECRETION OF LH IN PRIMARY CULTURES OF OVINE PITUITARY CELLS AND IN OVARIECTOMIZED EWES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

A NON-GENOMIC ACTION OF 17β-ESTRADIOL AS THE MECHANISM UNDERLYING THE ACUTE SUPPRESSION OF SECRETION OF LH IN PRIMARY CULTURES OF OVINE PITUITARY CELLS AND IN OVARIECTOMIZED EWES

The first objective of this study was to determine the ability of 17 β-estradiol (E_2) and conjugated forms of E_2, (E_2 conjugated to bovine serum albumin; E_2-BSA) and a novel synthesized conjugate (E_2 conjugated to a 15 amino acid sequence; E_2-PEP) to rapidly prevent the GnRH-induced secretion of luteinizing hormone (LH) and to determine the role of estradiol receptors (ERs) and ER subtypes in the mediation of the acute suppression of LH by E_2 in primary cultures of ovine pituitary cells. In the first experiment, cells were incubated for 15 min with 0 to 100 nM of E_2, E_2-BSA or E_2-PEP, plus 2 nM GnRH. In the second experiment, cells were treated with 100 nM of the following steroid hormones; progesterone (P_4), testosterone (T), hydrocortisone (HC), 17 α-estradiol (17 α-E_2), or the ERs antagonists tamoxifen (Tx), hydroxytamoxifen (HTx) or ICI 182,780 (ICI), with or without 1 nM of E_2, E_2-BSA or E_2-PEP, plus 2 nM GnRH. In the last experiment, cells were incubated with 0 to 100 nM of the selective ERα estrogen agonist (PPT), or the selective ERβ estrogen agonist (DPN) plus 2 nM GnRH. Relative to GnRH treated cells, 0.1 to 100 nM of E_2, E_2-BSA or E_2-PEP suppressed ($P < 0.01$) GnRH-induced secretion of LH. As expected incubation of pituitary cells with P_4, T, HC

or 17α-E_2 did not affect LH secretion when given alone, nor impaired the inhibition of E_2 on GnRH-induced secretion of LH ($P > 0.1$). Likewise, ER antagonists ICI, Tx or HTx did not affect ($P > 0.1$) secretion of LH when given alone, but they did prevent ($P < 0.01$) the inhibition of E_2 and conjugated forms of E_2 on GnRH-induced secretion of LH. Moreover, the selective ERα agonist but not the selective ERβ agonist decreased ($P < 0.01$) the GnRH-induced secretion of LH. The second objective was to determine how rapidly E_2 was able to suppress LH secretion in ovariectomized (OVX) ewes and to evaluate the ability of conjugated forms of E_2 to mimic the actions of E_2 on LH and follicle stimulating hormone (FSH) secretion. Animals ($n = 5$ or 6 per group) were infused for 4 h with 50 μg E_2 or equimolar concentration of E_2-BSA or E_2-PEP. LH and FSH were quantified from blood samples taken every 15 min from 4 h before to 5 h after the beginning of the infusion, and every h for the next 19 h. E_2, E_2-BSA, and E_2-PEP each induced an acute suppression in frequency of pulses of LH (< 20 min; $p < 0.01$). However, E_2, but not E_2-BSA or E_2-PEP, induced the characteristic pre-ovulatory like surge of LH (at 10 h after priming treatment), and a decreased in secretion of FSH (at 4 h after priming treatments). In conclusion, in ovine pituitary cells, E_2 and conjugated forms of E_2 prevented GnRH-induced release of LH in a steroid specific way. The inhibitory actions of E_2, E_2-BSA or E_2-PEP in GnRH-induced release of LH secretion were equally prevented by antagonistic compounds of ER, which suggest that ERs mediate this action. Moreover, the use of selective ER agonists indicates that acute inhibition of GnRH-induced release of LH by E_2 occurs via ERα. The acute inhibition of secretion of LH induced by E_2 in OVX ewes is not compatible with the classic genomic mechanism by which steroid hormones modulate cellular function. An acute, nongenomic action as the

mechanism underlying the sudden suppression in secretion of LH is supported by the fact that, conjugated forms of E_2 mimicked only the acute suppression of secretion of LH, without inducing the putative genomic actions of E_2 on secretion of LH or FSH. The presumed impermeability of conjugated forms of E_2 suggests that the plasma membrane is involved in mediating the acute effect of E_2. The ability of E_2-BSA and E_2-PEP to mimic only the acute action of E_2 justifies their further characterization as a tool for the study of acute, nongenomic plasma membrane-mediated actions of E_2.

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CHAPTER I

REVIEW OF LITERATURE

ACTIONS OF GnRH AND ESTRADIOL ON SYNTHESIS AND SECRETION OF GONADOTROPIN HORMONES

Introduction. Regulation of gonadotropin secretion by 17β-estradiol (E_2) has been examined extensively, however the mechanism underlying the rapid suppression of LH following administration of E_2 remains unknown. In the ewe and other mammalian species, E_2 has a biphasic effect on luteinizing hormone (LH) secretion, characterized by a decrease in LH secretion, followed by a pre-ovulatory surge of LH (Beck and Reeves, 1973; Nett *et al.*, 1984; Clarke and Cummins 1984; Clarke *et al.*, 1988; Herring *et al.*, 1991; Mercer *et al.*, 1993; Dhillon *et al.*, 1997; Molter-Gerard *et al.*, 2000). The mechanism by which E_2 exerts its effects has been traditionally considered to occur exclusively through regulation of transcription of target genes. However, in a wide variety of biological systems E_2 has been shown to regulate cellular function rapidly by nongenomic actions initiated at the plasma membrane (Katzenellenbogen, 1996; Simoncini *et al.*, 2003). Moreover, cellular function may be further modulated by interactions of the signaling pathways, activated via the plasma membrane, with the transcriptional machinery (Levin, 1999). However, to date there is no data generated in an animal model supporting an acute, nongenomic action of E_2.

Effects of GnRH on Gonadotropins. In the gonadotrope, binding of GnRH to its

seven transmembrane domain, G-protein coupled receptor, produces distinct physiological responses. These include secretion of LH and follicle stimulating hormone (FSH), synthesis of gonadotropin subunits, and up- and down-regulation of GnRH receptor (GnRHR) (Stojikovic and Catt, 1995a; Shupnik, 1996; Stanislaus *et al.*, 1998). The underlying mechanism by which GnRH stimulates this variety of physiological responses through a single class of receptors is not completely understood. Some studies have shown that the GnRHR is capable of coupling to multiple G-proteins (Stojikovic and Catt, 1992, Stojikovic *et al.*, 1994), which could affect distinct signaling pathways (Stanislaus *et al.*, 1998). In this regard, changes in frequency of GnRH pulses can differentially favor gonadotropin subunit and GnRHR synthesis (Kaiser *et al.*, 1995; Shupnik, 1996; Kaiser *et al.*, 1997a; Kaiser *et al.*, 1997b). In turn, expression of GnRHRs can be modulated by multiple endocrine inputs. In transiently transfected αT3-1 cells a tripartite enhancer of the murine GnRHR (mGnRHR) has been identified. The enhancer includes binding sites for steroidogenic factor-1 (SF-1), activating-protein 1 (AP-1) element, and GnRHR activating sequence (GRAS) (Duval *et al.*, 1997). In αT3-1 cells, the AP-1 element in the mGnRHR gene is a target of GnRH stimulation via at least two mitogen-activated protein kinase (MAPK) cascades, the extracellular signal-regulated kinase (ERK) 1/2 (White *et al.*, 1999) and the c-Jun NH_2-terminal kinase (JNK) pathways (Ellsworth *et al.*, 2003a; Ellsworth *et al.*, 2003b). Activation of either pathway stimulates binding of FOS/JUN family members to the AP-1 element (White *et al.*, 1999; Ellsworth *et al.*, 2003b). In αT3-1 cells, the GRAS element is responsive to activin/follistatin (Duval *et al.*, 1999). In transgenic mice the oGnRHR promoter

responds to GnRH and to E_2 (White *et al.*, 1999; Duval *et al.*, 2000).

There is compelling evidence to support inositol 1, 4, 5-trisphosphate ($InsP_3$)/Ca^{2+} in the signal transduction pathways involved in gonadotropin secretion induced by GnRH (for review see; Stojikovic and Catt, 1992, Stojikovic *et al.*, 1994; Stojikovic and Catt, 1995a; Stojikovic and Catt, 1995b; Kaiser *et al.*, 1997a; Kaiser *et al.*, 1997b; Naor *et al.*, 1998). In this model, binding of GnRH to its receptor induces conformational changes leading to activation of the heterotrimeric $G_{q/11}$ subfamily of G-proteins and phosphorylation of phospholipases. Phospholipase C (PLC), the more common lipase implicated in this system, hydrolyzes phosphatidylinositol 4, 5-biphosphate (PIP2), which generates diacylglycerol (DAG) and $InsP_3$ to stimulate release of intracellular Ca^{2+}. There is a biphasic pattern of intracellular Ca^{2+}, spike (release of intracellular Ca^{2+}) and plateau (influx of extracellular Ca^{2+}), that parallels hormone secretion. In turn, intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) activate protein kinase C (PKC), both of which synergizes to cause hormone release. Although early studies suggested that cAMP may be involved in the secretory response to GnRH (Adams *et al.*, 1979a), subsequent studies indicated that cAMP may mediate GnRH-induced synthesis of gonadotropin (Adams and Nett, 1979b; Conn et al., 1979; Liu and Jackson, 1981).

An interaction between E_2 and GnRH has been reported. In rat pituitary cells E_2 increases the GnRH-induced αLUC activity via PKC- and Ca^{2+}-dependent signaling pathways (Colin *et al.*, 1998). On the other hand, GnRH pulses increase levels of mRNA for estradiol receptor (ER) (Demay *et al.*, 1996) and nuclear E_2 binding sites in pituitary cells (Weisenberg *et al.*, 1979; Sing and Muldoon, 1983). In transiently transfected αT3-

1 cells, GnRH stimulated estrogen response element-containing promoters in an E_2-independent but ERα-dependent manner; whereas in rat pituitary cells, GnRH increased ERα transactivation compared with that in cells treated with E_2 alone, by a signaling pathway involving PKC and MAPK (Demay *et al.*, 2001). In this regard, it has been demonstrated that several kinases like PKC (Lahooti *et al.*, 1998), protein kinase A (PKA) (Schreihofer *et al.*, 2001; Coleman *et al.*, 2003), tyrosine kinase (Migliaccio *et al.*, 1996), and MAPK (Lu *et al.*, 2003) can activate ER, whereas protein phosphatase 2A (PP2A) dephosphorylates ERα (Lu *et al.*, 2003). Although, the biological importance of ER activation by GnRH has yet to be determined, it has been proposed that GnRH may contribute to maintenance of moderate levels of ERα activity in gonadotropes when estrogen levels are low (Demay *et al.*, 2001).

Effects of Estradiol on FSH. In ewes, E_2 induces a negative (Mercer *et al.*, 1993) or a biphasic (decrease and then increase; Jonas *et al.*, 1973; Reeves *et al.*, 1974) action on secretion of FSH. The initial decrease in secretion of FSH has been detected from 3 to 6 h after treatment with estrogens (Reeves *et al.*, 1974; Mercer *et al.*, 1989; Mercer *et al.*, 1993; Molter-Gerard *et al.*, 2000), remains low for 6 to 14 h, and is accompanied by a decrease in levels of FSHβ mRNA in the pituitary gland (Mercer *et al.*, 1989; Mercer *et al.*, 1993; Turzillo *et al.*, 1998a; Molter-Gerard *et al.*, 2000). These studies, carried out in OVX hypothalamo/pituitary-disconnected (HPD) ewes maintained with GnRH pulses (Mercer *et al.*, 1989; Mercer *et al.*, 1993; Turzillo *et al.*, 1998a) or in OVX ewes immunized against GnRH (Molter-Gerard *et al.*, 2000), indicate that the negative feedback effect of E_2 on secretion of FSH occurs at the level of the pituitary gland.

Similarly, in primary cultures of ovine pituitary cells, E_2 decreased secretion of FSH as early as 6 h after treatment, it remained low at 30 h (Miller *et al.*, 1977; Huang and Miller, 1980), and was accompanied by an inhibition in transcription of the FSHβ gene (Alexander and Miller, 1982; Shupnik, 1996; Baratta *et al.*, 2001). Apparently, the negative action of E_2 on expression of the FSHß gene in sheep is mediated by inhibition of pituitary β_B (Activin B) gene expression (Baratta *et al.*, 2001).

Effects of Estradiol on LH. E_2 exhibits a positive and negative feedback influence on secretion of LH both at the hypothalamus and the pituitary gland (Nett *et al.*, 1984; Mercer *et al.*, 1993; Herbison, 1998). E_2 microimplants positioned either in the medial preoptic area or caudal to the mediobasal hypothalamus in the vicinity of the arcuate nucleus of ewes decreased pulsatile secretion of GnRH 2 h after administration of E_2. This resulted in decreased secretion of LH preceding the pre-ovulatory surge of GnRH and LH, which occurred from 16 to 28 h after treatment with E_2 (Evans *et al.*, 1994; Caraty *et al.*, 1998). The rapid suppression of GnRH secretion may result from direct inhibition of electrical activity in GnRH neurons by E_2 (Herbison, 1998). Indeed, a direct hyperpolarizing influence of E_2 on guinea pig GnRH neurons has been demonstrated (Lagrange *et al.*, 1995).

There is a widespread disagreement about the effect of E_2 on expression of GnRH mRNA (Gore and Roberts, 1997). Apparently, E_2 does not alter content of GnRH mRNA in ewes (Dhillon *et al.*, 1997; Harris *et al.*, 1998), whereas in rats both stimulatory and inhibitory actions of E_2 on expression of GnRH mRNA have been reported (Petersen *et al.*, 1995; Zoeller *et al.*, 1998). It has been proposed that a differential effect of E_2 on

discrete sub-populations of GnRH neurons in the brain may explain this biphasic modulation of levels of GnRH mRNA (Herbison, 1998). In rat brain tissue, E_2 is capable of modulating signaling pathways and transcription factors; unfortunately, the biological significance of these data remain to be explained. For example, in an immortalized hippocampal cell line (Wade and Dorsa, 2003) and neurons of the preoptic area in ovariectomized (OVX) rats (Zhou *et al.*, 1996), E_2 rapidly induces phosphorylation of CREB, via both ERα and ERβ, a response mediated by ERK 1, but not PKA (Wade and Dorsa, 2003). In the same model, E_2 up-regulates α_{1B}-adrenergic receptors in hypothalamus and preoptic area (Karkanias *et al.*, 1996). In turn, stimulation of α_{1B}-adrenergic receptors activates PLC (Petitti and Etgen, 1991), which ultimately activates PKC (Nishizuka, 1992). Because of the apparent lack of ER in GnRH neurons, combined with their inability to concentrate E_2, it is thought that E_2 regulates secretion of GnRH through an intricate neurotransmitter network (Herbison, 1998).

The hypothalamus also seems to be a locus for the negative feedback of E_2 on gonadotropin secretion observed in long-term E_2-treated ewes and during the anestrus season (Karsch *et al.*, 1987; Karsch *et al.*, 1993). During anestrus, dopaminergic neurons mediate the negative action of E_2 on secretion of GnRH and this action has been related with an increase in FOS expression, tyrosine hydroxylase activity, and electrical activity of dopaminergic neurons (Gayrard *et al.*, 1994; Lehman *et al.*, 1996; Goodman *et al.*, 2000), as well as an increase in the percentage of ERα-positive neurons expressing FOS in the medial preoptic area (Stefanovic *et al.*, 2000). To further support the hypothalamus being the target for the suppressive action of E_2 on synthesis of gonadotropins, the

increase in the levels of all three gonadotropin subunit mRNAs observed after castration of rats, as well as the decrease in these after E_2 treatment (Shupnik *et al.*, 1989a) cannot be replicated in isolated pituitary tissue from OVX rats treated with E_2 (Shupnik *et al.*, 1989b). Therefore, E_2 does not appear to suppress synthesis of gonadotropins by a direct action at the pituitary gland. In contrast, E_2 may stimulate transcription of the rat (Shupnik and Rosenzweing, 1991) and bovine (Keri *et al.*, 1994) LHβ gene by a direct action on the pituitary gland. This may explain the ability of E_2 to induce secretion of LH in GnRH-deprived pituitary cells from anestrus ewes (Huang and Miller, 1980; Baratta *et al.*, 2001) and bovine pituitary cells (Padmanabhan *et al.*, 1978) cultured from 10 to 28 h. In mouse, however, treatment with E_2 in the absence of GnRH stimulation does not alter secretion of LH (Naik *et al.*, 1985).

The most common mechanism by which E_2 enhances pituitary sensitivity to GnRH is via an increase in the number of GnRHRs. A direct effect of E_2 on GnRHR number has been established *in vitro* using primary cultures of ovine (Gregg *et al.*, 1990; Laws *et al.*, 1990) and rat (Emons *et al.*, 1988; Quiñones-Jenab *et al.*, 1996) pituitary cells, as well as *in vivo* using OVX ewes infused with GnRH (Nett *et al.*, 1984) and OVX/HPD ewes maintained with GnRH pulses (Clarke *et al.*, 1988; Gregg and Nett, 1989; Turzillo *et al.*, 1995a; Kirkpatrick *et al.*, 1998a). The increase in number of GnRHRs occurs between 5 and 8 h after administration of E_2 (Nett *et al.*, 1984; Clarke *et al.*, 1988; Laws *et al.*, 1990; Gregg *et al.*, 1990), remains elevated for 24 h (Emons *et al.*, 1988; Kirkpatrick *et al.*, 1998a) after E_2 treatment, and is accompanied by an increase in the levels of GnRHR mRNA (Turzillo *et al.*, 1995a; Hamernik *et al.*, 1995; Kirkpatrick *et*

al., 1998a; Turzillo *et al.*, 1998a; Turzillo *et al.*, 1998b). When the ovine GnRHR promoter was transfected into the pituitary of mice, it retained its responsiveness to E_2 (Duval *et al.*, 2000), Therefore, enhancement on GnRH-induced secretion of LH in cultured pituitary cells induced by chronic treatment with E_2 (Padmanabhan *et al.*, 1978; Moss and Nett, 1980; Huang and Miller, 1980; Laws *et al.*, 1990), as well as the increase in pituitary content of LH (Padmanabhan *et al.*, 1978) may result from the stimulatory action of E_2 on expression of both GnRHR and LHß genes. In addition to the stimulatory input of E_2 on GnRHR number, the importance of removal of negative feedback induced by progesterone in frequency of GnRH pulses, and therefore in GnRHR mRNA expression has been well documented (Turzillo *et al.*, 1994; Turzillo *et al.*, 1995b; Turzillo *et al.*, 1998b; Kirkpatrick *et al.*, 1998b; Turzillo and Nett, 1999; Nett *et al.*, 2002).

One of the first demonstrations *in vivo*, for a rapid negative feedback effect of E_2 on LH secretion mediated at the level of pituitary gland, was obtained in OVX ewes infused with GnRH (Nett *et al.*, 1984). The pituitary loci as the site of the negative action of E_2 was corroborated by the use of OVX/HPD ewes maintained with GnRH pulses (Clarke *et al.*, 1988; Gregg and Nett, 1989; Mercer, *et al.*, 1993), OVX ewes immunized against GnRH (Molter-Gerard, *et al.*, 2000), and culture of bovine (Padmanabhan *et al.*, 1978), and rat (Tang and Spies, 1975; Emons *et al.*, 1988; Fallest and Schwartz, 1991) pituitary cells, as well as αT3-1 cells (McArdle *et al.*, 1992). Moreover, the *in vitro* studies showed that E_2 reduced secretion of LH by decreasing pituitary sensitivity to GnRH (Tang and Spies, 1975; Padmanabhan *et al.*, 1978; Emons *et al.*, 1988; Fallest and

Schwartz, 1991; McArdle *et al.*, 1992). In rats, however, a direct effect of E_2 on secretion of LH was detected (Fallest and Schwartz, 1991). Although experimental protocols were not specifically designed to examine rapidity of the negative feedback effect of E_2 on release of LH, a decrease in secretion of LH was detected within an hour after administration of E_2 in OVX and OVX/HPD ewes (Nett *et al.*, 1984; Gregg and Nett, 1989) and in cultured rat pituitary cells (Emons *et al.*, 1988). An acute suppression of GnRH-induced secretion of LH by E_2 has not been reported in cultured ovine pituitary cells. Because of the short time frame in which the decrease in circulating concentrations of LH occurred after treatment with E_2, Nett *et al.*, (1984) proposed that E_2 may mediate the suppression of LH secretion by a mechanism different from the classic genomic action. The fact that no changes in levels of LHβ mRNA (Mercer, *et al.*, 1993; Molter-Gerard, *et al.*, 2000) or pituitary content of LH (Clarke *et al.*, 1988) were observed during the decrease in secretion of LH induced by E_2 in ewes, further supports a non-transcriptional mechanisms mediating this effect. It is important to mention that discrepancies in the time frame required for E_2 to induce a stimulatory or inhibitory effect on the GnRH-induced secretion of LH have been seen in *in vitro* studies. Different experimental protocols (doses of E_2, length of E_2 pretreatment, or time points for data collection) and the physiological stage of the animal (proestrus *vs.* metestrus in rats, or anestrus *vs.* breeding season in ewes) may contribute to these discrepancies.

TRANSCRIPTIONAL ACTIVATION BY ESTRADIOL AND ESTROGEN RECEPTOR MODULATORS VIA ESTROGEN RECEPTORS ALPHA (ERα) AND BETA (ERβ)

Introduction. E_2 is a steroid hormone that displays an intriguing tissue-selective action modulating the function of many systems including the nervous system, the cardiovascular system, and the reproductive system, as well as others. The tissue-selective actions of E_2 were explained in the context of a wide array of effector components, such as growth factors and protein kinases, through which E_2-ER complex was linked to cell-specific transcriptional machinery to induce the classic genomic effects. The finding of a new ER subtype (named ERβ) in addition of the classic ER (now named ERα) together with the development of selective estrogen receptor modulators, and the increasing evidence for a nongenomic or non-transcriptional action of E_2, has altered our thinking about the tissue-selective actions of E_2. Both ER subtypes and the nongenomic action of E_2 are intrinsically related to the objective of this dissertation. In this section the role of ER subtypes on transcriptional modulation is discussed in the context of their function after binding of E_2 or selective estradiol receptor modulators (SERM). To present a more comprehensive discussion, a background on transcriptional modulation by steroid hormones, emphasizing modulation by E_2 is presented.

General Structure of Steroid Receptors. Estrogen receptors (ERs) are members of a large protein super family that includes receptors for other steroid hormones, retinoids, thyroid hormone, vitamin D3, and orphan receptors for which no ligand has been yet identified. These nuclear receptors function as ligand-activated transcription factors that

are activated by specific, high affinity binding of ligand to exert positive or negative effects on the expression of target genes (Katzenellenbogen *et al.*, 1996). Currently defined steroid receptors are proteins composed of distinct regions corresponding to functional and structural units called domains, labeled from A to F regions (Krust *et al.*, 1986). The A/B region contains a constitutively active activation function referred as transactivation function 1 (AF-1) that lies within the N-terminal domain. The C region is the DNA-binding domain (DBD), but other functions like receptor dimerization, nuclear localization, and heat shock protein (Hsp) 90 binding also have been attributed to this region. An important feature of this region is the presence of the "zinc finger" (two Zn^{2+} ions tetrahedrally situated within eight cysteines) that is critical for DNA binding specificity, DNA-dependent dimerization, and positive control of transcription (Freedman, 1992). The D region is a flexible hinge region between the DNA and ligand binding domains. This region is able to interact with transcription factors like c-Jun (Teyssier *et al.*, 2001), and part of this region is involved in DNA binding (Beato and Klug, 2000). The E region is the ligand binding domain (LBD), and like the DBD, is also involved in receptor dimerization, nuclear localization, binding of Hsp 90, and a ligand-activated transactivation function 2 (AF-2), that lies within the C-terminal domain. An important feature in this region is the helix 12, which operates as a "lid" after hormone has entered the binding pocket, generating new surfaces of contact with co-activators and activating AF-2 (Beato and Klug, 2000). The F region contains a C-terminal extension of the LBD. All these ER domains, with exception of region D, have been directly or indirectly implicated in discrimination of agonistic and antagonistic effects of synthetic

ligands (Montano *et al.*, 1995; Nichols *et al.*, 1998; Webb *et al.*, 1999).

Homology and Tissue Distribution of ERα and ERβ. In humans, ERα and ERβ are located in chromosomes 14 and 6, respectively (Enmark *et al.*, 1997). In the rat, the DBD and the LBD of ERβ is highly homologous to the corresponding domains of ERα (Kuiper *et al.*, 1996). However, the A/B, D, and F domains are poorly conserved between the two ER isoforms (Ruff *et al.*, 2000). Saturation analysis of recombinantly produced hERα and hERβ revealed a single binding component for E_2 with a high affinity constant (0.1 nM and 0.4 nM) for ERα and ERβ, respectively (Kuiper *et al.*, 1997). Tissue distribution and relative levels of mRNAs for ERα and ERβ have been estimated by *in situ* hybridization, immunohistochemistry, RNase protection assay, and RT-PCR. In some tissues a preferential localization of ER subtypes was observed, whereas co-localization of both ER subtypes was observed in others. Only a few of these studies have quantified ER proteins. In humans, mRNA for ERα and ERβ have been localized in the central nervous system, the immune system, the cardiovascular system, and bone, among others, while only ERα was detected in liver and only ERβ was localized in gastrointestinal tract (Gustafsson, 1999). Although uterus and mammary gland have both ER isoforms, ERα appears to be more abundant in these tissues (Gustafsson, 1999); ERβ is more abundant in urogenital tract (Hess *et al.*, 1997). In rats, moderate to high levels of mRNA for ERα were found in uterus, testis, pituitary, ovary, kidney, adrenal gland, and epididymis, while moderate to high levels of mRNA for ERβ were detected in prostate, ovary, lung, bladder, brain (cortex and cerebellum), testis, and uterus (Kuiper *et al.*, 1997; Kuiper *et al.*, 1998). In rat central nervous system more detailed studies have revealed a

differential distribution of ER subtypes. The vast majority of neurons have both ERα and ERβ mRNA in medial amygdala, preoptic area, stria terminalis, and bed nucleus (Shughrue *et al.*, 1997; Shughrue *et al.*, 1998b), and only few doubled-labeled cells are detected in arcuate and ventromedial nuclei (Shughrue *et al.*, 1998b). Some areas of the brain like paraventricular, supraoptic, and suprachiasmic nuclei, and pineal gland, express only ERβ mRNA (Shughrue *et al.*, 1997; Shughrue *et al.*, 1998b), and a high ERβ:ERα mRNA ratio is found in cerebral cortex (Shughrue *et al.*, 1997). Expression of ERβ protein has been corroborated in many of these regions (Li *et al.*, 1997; Price and Handa, 2000). In male and female sheep hypothalamus, the distribution of ERβ transcript was similar to that found in rat (Hileman *et al.*, 1999; Scott *et al.*, 2000), whereas co-localization of ERα and β transcripts was widely distributed including other hypothalamic regions (Scott *et al.*, 2000). Although, there appears to be a lack of ER immunoreactivity in GnRH neurons in hypothalamus of sheep (Lehman and Karsch, 1993; Herbison, 1995) and monkey (Herbison *et al.*, 1995), more recent studies, using multiplex RT-PCR (Skynner *et al.*, 1999) and non conventional immunocytochemistry (Butler *et al.*, 1999) reported the presence of ERα immunoreactivity and both ERα and ERβ mRNA in GnRH neurons. In sheep, ERs have also been localized in neurons containing the neurotransmitters gamma aminobutyric acid (Herbison, 1995), noradrenaline (Simonian *et al.*, 1998), β-endorphin (Lehman and Karsch, 1993), and somatostatin (Herbison, 1995). In pituitaries of adult female rats, expression of ERα mRNA is higher than that of ERβ (Wilson *et al.*, 1998; Shughrue *et al.*, 1998a). In sheep ER mRNA has been detected in pituitary (Madigou *et al.*, 1996) and ERs have been co-

localized in cells immunoreactive for LH from the ventral portion of the pars tuberalis (Lehman and Karsch, 1993). However, the presence of ER subtypes and their relative expression remains to be established.

Isoforms of mRNA for Estrogen Receptors. Isoforms of ER mRNAs are thought to arise from alternative splicing or single nucleotide alterations of the primary ER transcript. A number of isoforms of mRNA for ER have been described, particularly in cancer cell lines or tumors (Pfeffer *et al.*, 1996). Sequencing of ER transcripts revealed the lack of one or more exons, which prevented E_2-dependent transactivation in most isoforms (Hoshino *et al.*, 1995). In tumor cell lines, two ERα transcripts, ERΔ5 (Ohlsson *et al.*, 1998) and ERΔ7 (Pedrero *et al.*, 2003) have been reported as dominant negative receptors in the presence of the full-length ER subtypes. In fetal and newborn rat pituitaries three ERα mRNA isoforms ERΔ3, ERΔ4, and ERΔ3/4, have been detected and their transcripts have been translated *in vivo* (Pasqualini *et al.*, 1999). There is an isoform-dependent differential modulation of transcription through ERE (Pasqualini *et al.*, 2001). Contrary to that found for ERα isoforms, rat and human ERβ transcripts have been detected in a variety of tissues like testis, ovary, thymus, prostate, pituitary, muscle, and the nervous system (Petersen *et al.*, 1998; Ogawa *et al.*, 1998; Hanstein *et al.*, 1999; Richard *et al.*, 2000). In rat, at least five isoforms of ERβ mRNA have been reported, which include the original ERβ (now termed ERβ1 or ERβ1s), and the variants ERβ2, ERβ1δ3 and ERβ2δ3, and ERβ1δ4. All five ERβ transcripts have been efficiently transcribed and translated (Petersen *et al.*, 1998; Hanstein *et al.*, 1999; Richard *et al.*, 2000). Relative levels of ERβ1 and ERβ2 transcripts are similar in pituitary, ovary,

prostate, and muscle (Petersen *et al.*, 1998) Characterization of the two main receptors (β1 and ß2) in transfected cells showed that both receptors bind to estradiol response elements (EREs), heterodimerize with each other as well as with ERα. 4-OH-tamoxifen competed equally well for E_2 binding on both ERβ1 and ERβ2 receptors; functional differences, however, may define their physiological actions. For example, ERβ1 receptor has a binding affinity similar for E_2 and an E_2-dependent transactivation similar to ERα receptor; but a low binding and a weak E_2-dependent transactivation was detected for ERβ2 (Petersen *et al.*, 1998; Hanstein *et al.*, 1999). Although the differential intracellular localization of some E_2-responsive ER isoforms suggests a potential role of ERß isoforms in the mediation of acute actions induced by E_2 in mature animal tissue, to date no functional role has been attributed to these transcripts. Recently, a distinct plasma membrane-associated ER transcript has been reported in immature neocortical tissue of ERα gene disrupted (ERKO) mice. This receptor is responsive to E_2 and is differentially modulated by SERM (Toran-Allerand *et al.*, 2002).

Transcriptional Regulation by Steroid Hormone-Steroid Receptor Complex. It is believed that E_2, like other lipophilic steroid hormones, enters the cell by simple diffusion and binds within the cytoplasm, to a multiprotein complex of chaperones like Hsp90 and to the LBD on the ER. Hormone binding induces an E_2-ER complex transformation, leading to loss of chaperone proteins and activation of AF-2, which increases affinity for an ERE. The resulting transcription pre-initiation complex relays activating or repressing signals to the transcriptional machinery of target genes (Beato and Klug, 2000). In addition to the SRE-mediated effects, the SR controls promoter activity through a direct

or indirect interaction with nuclear proteins. These proteins have been classified into; a) general transcription factors (GTFs) that direct low level transcription, b) chromatin factors involved in nucleosome disruption, c) co-factors (co-activators or co-repressors) also named transcription intermediary factors (TIFs), which function as a bridge between SR and/or other nuclear proteins, to enhance or reduce gene transcription, and d) DNA-bound sequence-specific transcription factors, which in turn act as a link between co-factors and DNA sequences (Beato and Sanchez-Pacheco, 1996). The prevailing idea in the formation of the transcription initiation complex is that GTFs, assembled in an ordered fashion, will determine RNA polymerase specificity via a process where interactions with SR is mediated by multiple TIFs. Members of steroid receptor co-activator-1 (SRC-1; Onate *et al.*, 1995) and SRC-3 (Ogryzko *et al.*, 1996; Spencer *et al.*, 1997) have been identified as enhancers of transcriptional activation by ERα. The AF-1 on the ER displays little hormone dependent transactivation (Webb *et al.*, 1998), however, it can interact with co-activators and synergizes with AF-2 (Sadovsky *et al.*, 1995). Between the DNA-bound sequence-specific transcription factors mediating ER signal transduction are the AP-1 complex and JUN/FOS family member of oncoproteins (Zhu and Pfaff, 1998; Webb *et al.*, 1999), which interact with DNA as homodimers (JUN/JUN) or heterodimers (JUN/FOS; Teyssier *et al.*, 2001). These proto-oncoproteins may stimulate or inhibit transcriptional activation in a cell-specific context (Katzenellenbogen, 1996). Study of mechanism(s) of transcriptional activation by E_2 and SERMs have been carried out in transfection systems using different cell lines like COS-1, MCF-7, TSA-201, HEC-1, Ishikawa cells, CHO, HeLa, GT1-1, MDA-MB-231, as

well as in transgenic mice, and in rat pituitary and uterine cells. These cells are transfected with vectors containing the full length wild type ER, or with specific ER domains, with or without point mutations, and vectors containing a reporter gene linked to an ERE or AP-1 consensus sequence. The classic pathways of transcriptional activation by E_2-ER complex occur via EREs or AP-1 consensus sequences (Philips *et al.*, 1993; Webb *et al.*, 1995; Uht *et al.*, 1997; Zhu and Pfaff, 1998; Webb *et al.*, 1999; Duval *et al.*, 2000; Jakacka *et al.*, 2001). However, ER may interact with gene regulatory sites through non-consensus elements that may exist as single or multiple, full or half sites (Porter *et al.*, 1997; Katzenellenbogen and Katzenellenbogen, 2000; Cheng *et al.*, 2003) or with other sites like serum response elements (Jager *et al.*, 2001). For example, in the pituitary of transgenic mice an E_2-responsive ovine GnRHR promoter (Duval *et al.*, 2000) and an E_2-responsive rat neurotensin promoter (Watters and Dorsa, 1998) do not contain a canonical ERE. The jun/fos proto-oncogene family members are important targets of E_2 (Weisz and Rosales, 1990; Moenter *et al.*, 1993; Stefanovic *et al.*, 2000). Up-regulation of jun/fos genes results in transcriptional enhancement of other E_2 target genes by the binding of JUN/FOS proteins to ERE and recruitment of other transcription factors like p160s, to the JUN/FOS complex (Webb *et al.*, 1999). Finally, ER can also be activated in a ligand-independent manner by other signaling molecules like epidermal growth factor (EGF) (Ignar-Trowbridge *et al.*, 1993; Bunone *et al.*, 1996), cAMP (Aronica and Katzenellenborgen, 1993) and insulin-like growth factor-I (IGF-I) (Lee *et al.*, 1999; Martin *et al.*, 2000).

Transcriptional Regulation by E_2 Via ERα and ERβ. Some structural and

functional differences between ERα and ERβ subtypes have been determined. Human ERα contains two distinct transactivation domains, a weak AF-1 and a strong hormone-dependent AF-2, whose transcriptional activity is influenced by cell and promoter context. Human ERβ also contains an AF-2, but it does not contain the constitutive binding site for GRIP1 co-activator that lies at the AF-1 domain in the ERα (Webb *et al.*, 1999). From a functional point of view, E_2-ERα activates gene transcription by both the classic ERE and the AF-mediated AP-1 pathways (Webb *et al.*, 1995; Peach *et al.*, 1997; Webb *et al.*, 1999), while E_2-ERβ activates gene transcription by the classic ERE pathway, but transcription is inhibited by the ERβ AP-1 mediated pathway (Peach *et al.*, 1997; Webb *et al.*, 1999; Hall and McDonnell, 1999). Although, it has been proposed that ERβ functions as an inhibitor of ERα transcriptional activity induced by E_2 (Hall and McDonnell, 1999), it may be highly dependent on cell type. An interaction between ER isoforms is plausible since both, ERα and ERβ, can bind to the palindromic response element as homo- or herterodimers (Pettersson *et al.*, 1997; Ogawa *et al.*, 1998; Hall and McDonnell, 1999; Tremblay *et al.*, 1999) with similar DNA binding affinity between α-homo- and heterodimers, both of which bind with greater affinity than β-homodimers (Cowley *et al.*, 1997). Therefore, the cell-specific transcriptional machinery by which E_2 exerts its wide range effects, the existence of more than one ER subtype, their tissue distribution, and the relative level of the two isoforms are key determinants of cellular response to E_2 (Katzenellenbogen *et al.*, 1996). Development of synthetic ER ligands with differential action (agonistic or antagonistic), affinity, and potency brought new interest in characterizing the roles of ER subtypes.

Characterization and Transcriptional Regulation of ER Modulators. The terms, agonist and antagonist of E_2 define the capability of synthetic ER ligands to mimic or prevent, respectively, the actions of E_2 in target cells; "partial" agonist refers to lower maximal increases in transcriptional activity induced by some synthetic ligands (Barkhem *et al.*, 1998). Characterization of ER modulators involves determination of the potential biological activity, estimated as the relative estrogenic potency (REP) or the relative anti-estrogenic potency (RAP), and estimation of the relative binding activity (RBA) of the ligand to the receptor. Table 1 lists the RBA for ERα and ERβ and as an index of receptor binding selectivity, the selective RBA ratio between ERα and ERβ of SERMs and different steroid hormones. The REP and the selective REP ratio between ERα and ERβ of agonistic compounds on reporter gene expression are presented in Table 2, while the relative anti-estrogenic potency (RAP) and the selective RAP ratio between ER subtypes of preferential antagonistic ligands and a pure antagonistic compound are presented in Table 3. Finally, the ER selectivity of partial agonistic ligands is listed in Table 4. In the studies discussed here, the RBA was determined using uterine cell homogenates (Labrie *et al.*, 2001) or the full length of ERα and ERβ proteins expressed in different recombinantly produced cell lines (Kuiper et al., 1997; Sun *et al.*, 1999). The REP and the RAP were determined using the full length clones of ERα and ERβ proteins in different cell lines transfected with reporter plasmids. These plasmids included an ERE (Sun *et al.*, 1999; Meyers *et al.*, 2001), together with several promoters of E_2 target genes (Barkhem *et al.*, 1998), or in a range of ER responsive gene sites like non-consensus EREs, multiple half-EREs, AP-1, and sites where ER represses gene transcription

(Harrington *et al.*, 2003). As expected, nonestrogenic steroid hormones like progesterone, testosterone, and hydrocortisol do not bind to ERs (Table 1). Although the transcriptionally inactive stereoisomer 17α-E_2 had a RBA and a REP higher than several others ER modulators, traditionally it has been considered a criterion for stereo specificity (Noteboom and Gorski, 1965; Kuiper *et al.*, 1997). With few exceptions, the REP of pure agonistic ligands and the RAP of preferential or pure antagonistic compounds were several orders of magnitude lower than their respective RBA. This suggests that factors beyond ligand-receptor interaction such as receptor-co-activator interactions and the *in vitro* system utilized are important determinants of transcriptional potency (Sun *et al.*, 1999). In pure agonistic compounds, the higher ER selective RBA the higher ER selective REP was found (Table 1 and 2). The ER agonist propyl pyrazole triol (PPT) presented not only the highest, but also an exclusive ERα selective agonism on reporter gene transcriptional expression. Moreover, PPT recruited co-activators (SRC-1, Grip-1, and SCR-3) through ERα but not through ERβ. E_2 and others less ER selective agonists recruited similar levels of co-activators via both ERα and ERβ (Kraichely *et al.*, 2000). Although not as selective as PPT, 2,3-bis (4-hydroxyphenyl) propionitrile (DPN) showed the highest ERβ selective agonism followed by genistein (Table 2). R, R-enantiomer *cis*-diethyl-substituted tetrahydrochrysene (R, R-THS) showed a unique characteristic, inducing an agonistic effect via ERα and an antagonistic effect via ERβ (Table 2 and 3). Although the actions of R, R-THC appear to be promoter-dependent (Harrington *et al.*, 2003), the ERα agonistic selectivity was further corroborated by the fact that R, R-THC recruits co-activators via ERα but not via ERβ (Kraichely *et al.*,

2000). Unlike pure agonistic compounds like PPT and DPN, synthetic ER ligands with mixed actions (partial agonist/preferential antagonist) like tamoxifen and 4-OH-tamoxifen bind to either ERα or ERβ with similar RBA (Table 1). The antagonistic actions of these compounds are mediated by both ERα and ERβ (Table 3); whereas the partial agonism appears to be mediated by ERα (Table 4). Several reports indicate that the ERβ-mediated antagonistic action, induced by synthetic ligands, occurs via the ERE pathway (Peach *et al.*, 1997; Barkhem *et al.*, 1998; Webb *et al.*, 1999; Hall and McDonnell, 1999; Katzenellenbogen and Katzenellenbogen, 2000), whereas the AP-1 pathway is involved in the mediation of agonistic effects (Peach *et al.*, 1997). On the other hand, in most studies the ERα-ERE pathway is involved in mediating the partial agonistic actions by these compounds, while in a promoter- and cell-dependent context the ERα-AP-1 pathway has been reported to function in an agonistic or an antagonistic manner (Uht *et al.*, 1997; Peach *et al.*, 1997; Webb *et al.*, 1999; Tremblay *et al.*, 1999; Hall and McDonnell, 1999; Katzenellenbogen and Katzenellenbogen ., 2000; Jakacka *et al.*, 2001; Harrington *et al.*, 2003). Most studies on the mechanism by which ER synthetic ligands antagonize the effects of E_2 have focused on tamoxifen and 4-OH-tamoxifen. In general, it is accepted that they act as competitive inhibitors of E_2 binding at the ER (Wakeling *et al.*, 1984; Jordan, 1993). In a promoter context, tamoxifen and 4-OH-tamoxifen prevent AF-2 activation (Tzukeman *et al.*, 1994; Wakeling *et al.*, 1995) whereas AF-1 remains active (Tzukeman *et al.*, 1994). When gene transcription is activated by these synthetic ligands, it results in recruitment of co-repressor (Katzenellenbogen and Katzenellenbogen, 2000).

Table 1. Relative binding affinity (RBA)[a] and selective RBA ratio of ER synthetic ligands and steroid hormones [b]

Ligand	ERα	ERβ	α:β	β:α	effect [c]	Source
17β-estradiol	100	100	1	1	Ag	All quotes
Diethylstilbestrol	468	295	1.6		Ag	Kuiper (1997)
Genistein	5	36		7	Ag	Kuiper (1997)
Genistein	0.7	13		19	Ag	Meyers (2001)
17α-estradiol	1	?				Hajek (1997)
17α-estradiol	58	11	5			Kuiper (1997)
Testosterone	<0.0001	<0.001	1	1		Kuiper (1997)
Progesterone	<0.0001	<0.0001	1	1		Kuiper (1997)
Corticosterone	<0.0001	<0.0001	1	1		Kuiper (1997)
PPT	49	0.12	408		Ag	Stauffer (2000)
DPN	0.25	18		72	Ag	Meyers (2001)
Pyrazole	60	18	3		Ag	Sun (1999)
R, R-THC	3.6	25		7	pAg/Ant	Sun (1999)
Tamoxifen	7	6	1.2		pAg/Ant	Kuiper (1997)

4-OH-Tamoxifen	178	339		1.9	pAg/Ant	Kuiper (1997)
ICI 164,384	85	166		2	pAg/Ant	Kuiper (1997)
ICI 182,780	32	25	1.3		Ant	Sun (1999)
ICI 182,780[d]	115	115	-	-	Ant	Labrie (2001)

[a] RBA = IC_{50} (E_2) /IC_{50} (test compound) X 100. RBA for E_2 is 100%.
[b] Competitive binding assays were made using the full length or the ligand binding domain of cloned ERα and ERβ.
[c] Ag = agonist, pAg = partial agonist, Ant = antagonist.
[d] Competitive binding assay was carried out using uterine cells homogenate, therefore ER selectivity was not determined.

Table 2. Relative estrogenic potency (REP) [a] and selective REP of ER synthetic ligands [b]

ligand	ERα	ERβ	α:β	β:α	source
17β-estradiol	100	100	1	1	All quotes
Diethylstilbestrol	25	50		2	Barkhem (1998)
Genistein	0.13	2.22		17	Barkhem (1998)
Genistein	0.09	0.65		7	Meyers (2001)

17α-estradiol	0.42	0.8		2	Barkhem (1998)
PPT	~10	NR [c]	full α		Stauffer (2000) Kraichely (2000) Harrington (2003)
DPN	0.027	4.6		170	Meyers (2001)
Pyrazole	~2	~0.0167	120		Sun (1999) Harrington (2003)
R, R-THC	~0.07	NR	full α		Sun (1999) Kraichely (2000) Harrington (2003)

[a] REP = EC_{50} (E_2) / EC_{50} (test compound) X 100. REP for E_2 is 100%.
[b] Reporter gene expression was determined in different *in vitro* systems, using the full length or the ligand binding domain of cloned ERα and ERβ.
[c] NR = no response even at :M concentration.

Table 3. Relative anti-estrogenic potency (RAP) [a] and selective RAP of ER synthetic ligands [b]

ligand	ERα	ERβ	α:β	β:α	Source
R, R-THC	NR [c]	~0.1			Sun (1999) Harrington (2003)
Raloxifene	1.25	0.35	4		Barklem (1998)
Tamoxifen	0.0125	0.05		4	Barklem (1998)
4-OH-Tamoxifen	0.313	0.833		3	Barklem (1998)
ICI 164,384	1	6.7		7	Barklem (1998)
ICI 182, 780 [c]	23	23	-	-	Labrie (2001)

[a] RAP = $IC_{50}(E_2)$ / IC_{50} (test compound) X 100. E_2 potency is 100%.
[b] Inhibition of reporter gene expression was determined in different *in vitro* systems, using the full length or the ligand binding domain of cloned ERα and ERβ.
[c] Determined as the capability of ICI 182,780 (IC_{50} = 0.434 nM) to suppress cell proliferation induced by 9-day exposure to 0.1 nM E2. ER selectivity was not determined in this experiment.

Table 4. ER selective partial agonistic action of ER synthetic ligands, expressed as percentage of maximum E_2 response [a]

ligand	ERα	ERβ	source
Tamoxifen	9	NA	Barkhem (1998)
4-OH-Tamoxifen	13	NA	Barkhem (1998)
ICI 164,384	3	NA	Barkhem (1998)
Raloxifene	12	NA	Barkhem (1998)

[a] Reporter gene expression was determined in different *in vitro* systems, using the full length of cloned ERα and ERβ.
[b] NA = no agonistic response.

Regarding to the pure antagonist ICI 182,780, multiple changes in ER function appear to contribute to the complete abrogation of the transcriptional action of E_2 (Wakeling, 2000). These include impaired dimerization (Chen *et al.*, 1999), increased receptor degradation (Borras *et al.*, 1996), and disrupted nuclear localization (Dauvois *et al.*, 1993) Likewise, ICI 182,780 failed to recruit co-activators (Kraichely *et al.*, 2000) and prevented E_2-induced expression of SRC-1 through both ERα and ERβ (Labrie *et al.*, 2001). The acute- and long-term biological actions of ER modulators are discussed in other sections of this chapter. As suggested by the previous discussion, the final action of ligand-ER complex is determined not only by the ER subtype involved, but also by the nature of the ligand. A dramatic example is seen with ERβ, which activates (via AP-1 pathway) or prevents (via ERE pathway) transcription when it binds to a synthetic ligand. These actions, however, are completely inverted when ERβ binds to E_2 (Peach *et al.*, 1997).

MOLECULAR BASIS OF THE ACUTE ACTIONS OF E_2 ON CELLULAR FUNCTION

Introduction. This section reviews signaling pathways acutely modulated by E_2, followed by separate discussions about the techniques used to characterize the acute, nongenomic actions of E_2 and the nature of plasma membrane receptors involved in mediating actions of E_2. During the last few years an overwhelming number of reports have demonstrated that E_2 rapidly, in seconds to minutes, modulates cell function by nongenomic mechanisms. In several cellular processes, genomic actions of E_2 are seen as a consequence of signaling initiated at the level of plasma membrane. An increasing number of signaling pathways, traditionally considered exclusive for protein hormones, as well as cell membrane ion channels, have been identified as mediators of E_2 action in a number of model systems including tumoral and non-tumoral cell lines, and primary cell cultures. Acute actions of E_2 include activation of small G-proteins like Ras, heterotrimeric G-proteins, PKC, PKA, PLC, phosphatidylinositol 3-kinase (PI3K), nitric oxide synthase (NOS), tyrosine kinases, MAP kinase, and ion channels. The role of specific elements in the signaling cascade has been examined by using specific inhibitors or activators of these proteins, and by transfection of dominant negative or constitutively active proteins. The steroid-specificity of the acute actions of E_2 on cellular processes is usually tested by challenging cells with other steroid hormones. Further, acute actions of E_2 have been mimicked using E_2-BSA conjugates; this has been interpreted as an effect initiated at the plasma membrane. A functional interaction of ER with other plasma membrane receptors, resulting in signal transduction, further supports the initiation of

signaling at the level of plasma membrane. In many of these studies, investigators have recognized that the acute actions of E_2 occur via ERs, putatively located in or associated with the plasma membrane, and in some cellular processes a response dependent on specific ER subtypes has been identified. Recognition of ER as a fundamental element in the mediation of the acute actions induced by E_2 has been evaluated by the use of anti-estrogenic compounds, and antibodies against ER. In addition, labeling studies using ER antibodies and E_2-BSA have demonstrated E_2 binding sites on the plasma membrane. The identity of membrane ERs has been explored by means of transfection of cDNA encoding for nuclear ER (ERα or ERβ) in cells not expressing ER. Recently, new ER modulators have proved to activate transcription in a selective ER subtype manner, thus resulting in another potential tool to identify the ER subtype involved in the acute actions of E_2. Additionally, some investigators have postulated the existence of ER distinct from ERα and ERβ subtypes (Singh *et al.*, 1999; Singh *et al.*, 2000; Toran-Allerand, 2000; Nethrapalli *et al.*, 2001; Toran-Allerand *et al.*, 2002).

Modulation of Nitric Oxide Synthase (NOS) by E_2. Over the past decade an interaction among E_2, GnRH, and the gaseous neurotransmitter nitric oxide (NO) in the control of hypothalamic-pituitary function has been documented in rats (Ceccatelli *et al.*, 1996; Garrel, *et al.*, 1998; McCann *et al.*, 1999). Most of these studies were carried out using NO competitive inhibitors, NO scavengers, NO donors, and NOS inhibitors. The best characterized role of NO is to stimulate GnRH release from hypothalamus and the GT1 cell line (a hypothalamic GnRH-secreting neuronal cell), where both the neuronal (n) and the endothelial (e) isoforms of NOS have been implicated in a mechanism that

involves guanylyl cyclase activation (Moretto *et al.*, 1993; Mahachoklertwattana *et al.*, 1994; Canteros *et al.*, 1996; Lozach *et al.*, 1998; McCann *et al.*, 1999; Prevot *et al.*, 2000; Knauf *et al.*, 2001;Barnes *et al.*, 2001 Karanth *et al.*, 2004). One physiological function of NO is to mediate GnRH release induced by several neurotransmitters like N-Methyl-D-aspartate (NMDA) (Mahachoklertwattana *et al.*, 1994; Barnes *et al.*, 2001 Karanth *et al.*, 2004), glutamic acid (Karanth *et al.*, 2000) and norepinephrine (Canteros *et al.*, 1996). Activation of NOS that preceded the NMDA-induced release of GnRH is dependent on extracellular Ca^{2+} levels (Mahachoklertwattana *et al.*, 1994). NO/cGMP also mediates the decrease in GnRH synthesis induced by glutamate in GT1 cells (Belsham and Mellon, 2000). In rat pituitary cells, incubation for 4 hr with SNP, a NO donor, decreased GnRH-induced release of LH (Ceccatelli *et al.*, 1993). GnRH has a stimulatory action on nNOS mRNA and protein expression in rat pituitaries (Garrel, *et al.*, 1998), whereas in hypothalamus E_2 enhances nNOS mRNA (Ceccatelli *et al.*, 1996) and acutely increases NO production, resulting in GnRH release via guanylyl cyclase (Prevot *et al.*, 1999). The increase in nNOS mRNA and protein expression in rat pituitaries after OVX (Ceccatelli *et al.*, 1993; Garrel, *et al.*, 1998) and its prevention by treatment with E_2 (Garrel, *et al.*, 1998) is thought to be a consequence of the negative feedback of E_2 on GnRH secretion. A direct action of E_2 on NOS activity at the level of the pituitary gland has not been documented.

The importance of E_2 as a vasoprotective and an atheroprotective agent has been demonstrated recently (Farhat *et al.*, 1996). This vasoprotective effect of E_2 is at least partially mediated by activation of eNOS, resulting in NO production (Chambliss and

Shaul, 2002a). E_2 has both short- and long-term effects on eNOS function. Treatment with E_2 for several days in rabbits (Gisclard *et al.*, 1988) and ewes (Veille *et al.*, 1996) or for several months in female monkeys (Williams, *et al.*, 1990) enhanced eNOS expression resulting in vasolidation. In cultured ovine endothelial cells E_2 enhanced eNOS mRNA and protein levels within hours (MacRitchie *et al.*, 1997) by a mechanism not involving the classical ERE-mediated process (Kleinert *et al.*, 1998). Furthermore, down-regulation of eNOS by TNFα, due to enhanced degradation of eNOS mRNA in cultured endothelial cells, is prevented by E_2 in an ER-dependent manner (Sumi *et al.*, 2001). The bone protective and the antiapoptotic effects of E_2, which occurs via down-regulation of TNFα, are mediated via JNK inactivation (Srivastava *et al.*, 1999; Razandi *et al.*, 2000a; Razandi *et al.*, 2000b). Therefore, blocking the MAP kinase pathway by E_2 may enhance eNOS expression. An acute, non-transcriptional activation of eNOS by E_2 has been detected in cultured endothelial cells after incubation with E_2 for 5 to 15 min (Chen *et al.*, 1999; Kim *et al.*, 1999; Russell *et al.*, 2000; Selles *et al.*, 2001). Multiple signal transduction pathways have been involved in the rapid E_2-induced activation of eNOS, including G-protein (Wyckoff *et* al., 2001), cGMP (Russell *et al.*, 2000), tyrosine kinase (Chen *et al.*, 1999; Simoncini *et al.*, 2003), mitogen-activated protein kinase kinase-1 (MEK-1) (Chen *et al.*, 1999), ERK 1/2 (Russell *et al.*, 2000; Hisamoto *et al.*, 2001a;Wyckoff *et* al., 2001; Simoncini *et al.*, 2003), and PI3-kinase-protein kinase B/Akt pathway (Haynes *et al.*, 2000;Hisamoto *et al.*, 2001a; Hisamoto *et al.*, 2001b; Simoncini *et al.*, 2003). In addition, E_2-induced activation of Akt and eNOS appears to be Ca^{2+} dependent (Goetz *et al.*, 1999; Kim *et al.*, 1999; Chambliss *et al.*, 2000; Hisamoto *et al.*,

2001b) and requires both intracellular and extracellular Ca^{2+} (Kim *et al.*, 1999; Hisamoto *et al.*, 2001b). However, E_2-induced activation of eNOS can occur independently of Ca^{2+} mobilization (Chambliss *et al.*, 2000; Chen *et al.*, 2003).

Modulation of Protein Kinase Pathways by E_2. Estradiol rapidly modulates at least three mitogen-activated protein (MAP) kinase pathways, represented by the down-stream kinases, c-Jun N-terminal kinase (JNK), p38α/β, and extracellular-signal regulated kinases (ERK) 1/2 (Pearson *et al.*, 2001), as well as other protein kinases and tyrosine kinases. In turn, kinases up- or down regulate gene transcription by phosphorylation of cell-specific transcription factors (Morey *et al.*, 1998; Rowan *et al.*, 2000; Razandi *et al.*, 2000a; Wade *et al.*, 2003). Although a rapid action of E_2 on JNK activity has been only shown in Chinese hamster ovary (CHO) cells (Razandi *et al.*, 1999), the bone protective (Srivastava *et al.*, 1999) and the antiapoptotic (Razandi *et al.*, 2000b) effects of E_2 are due to blockadge of JNK activity. In bone, inactivation of JNK prevents phosphorylation of JUN proteins, decreases tumor necrosis factor (TNF)-α gene expression, and therefore decreases bone remodeling. In MCF-7 inactivation of JNK prevents phosphorylation of the active-non-phosohorylated forms of antiapoptotic proteins. In endothelial cells (EC), E_2 activates p38β, but inhibits p38α (Razandi *et al.*, 2000a). The rapid activation of the p38β MAP kinase pathway by E_2 (Razandi *et al.*, 2000a; Razandi *et al.*, 2002) occurs via mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2) upstream, resulting in phosphorylation of heat shock protein 27 (Hsp 27), a transcription factor involved in prevention of hypoxia-induced apoptosis (Razandi *et al.*, 2000a).

The rapid E_2-induced phosphorylation of ERK 1/2 is involved in many cellular processes, for example neuroprotection against glutamate toxicity (Singer *et al.*, 1996; Singer *et al.*, 1999; Mize *et al.*, 2003), heart-protection (Jager *et al.*, 2001), adipose tissue metabolism (Santos *et al.*, 2002), prolactin gene expression (Watters *et al.*, 2000), cell proliferation and tumor progression (Migliaccio *et al.*, 1996; Wade *et al.*, 2001; Song *et al.*, 2002), anti-apoptosis (Razandi *et al.*, 2000a), cell death (but not via apoptosis) of immature cerebral neurons (Wong *et al.*, 2003), secretion of prostaglandins (Pedram *et al.*, 2002), and other yet to be established processes (Wade *et al,*. 2003). Moreover, E_2 is also able to decrease angiotensin II-induced ERK phosphorylation, resulting in a decrease of endothelin-1 secretion from EC, which may participate in the cardiovascular-protective effect of E_2 (Morey *et al.*, 1998). From these and other studies, different elements upstream of ERK 1/2 have been identified and the following general cascade has been proposed: E_2-ER/Src (a non-receptor tyrosine kinase)/Shc-Grb2 (adapter protein)/Sos (son of sevenless; a guanine nucleotide exchange protein) complex/Ras (a monomeric G protein)/Raf/MAPK (upstream protein kinase family members)/ERK 1/2.

Besides this general model, other upstream proteins are acutely activated by E_2. In white adipocytes, E_2-ER complex phosphorylates ERK 1/2 through activation of both, Src/ phosphatidylinositol 3-kinase (PI3K)/Ras/Raf/MEK and PKC/Raf/ MEK, resulting in ERK-dependent phosphorylation of cAMP response element-binding protein (CREB; Santos *et al.*, 2002), a mechanism known to involve G-proteins. In hippocampal cells E_2-induced activation of ERK 1/2 promoted CREB phosphorylation within 15 min, in a PKA-independent pathway (Wade *et al.*, 2003). Rapid protein kinase phosphorylation by

E_2 also occurs in the PI3K/Akt pathway, which stimulates the secretion of PGI_2 and PGE_2, and up-regulates gene expression within 40 to 60 min in EC (Pedram *et al.*, 2002). In several cells types like rat hypothalamic cells, neuronal cell lines, rat uterine cells, MCF-7 cells, and the rat and mouse models, E_2 rapidly activates elements of the adenylate cyclase/cAMP/PKA pathway, resulting in CREB phosphorylation (Aronica *et al.*, 1994; Zhou *et al.*, 1996; Watters and Dorsa, 1998). These effects were independent of *de novo* protein or mRNA synthesis (Aronica *et al.*, 1994; Watters and Dorsa, 1998), and the E_2-dependent activation of cAMP/PKA resulted in neurotensin gene expression, a GnRH stimulatory neurotransmitter in mouse brain (Watters and Dorsa, 1998). There is bidirectional cross-talk between ER and plasma membrane tyrosine kinase receptors, like epidermal growth factor receptor (EGFR) and insulin-like growth factor I receptor (IGFR-I; Levin, 2003). E_2-ERα rapidly stimulates EGFR-dependent ERK phosphorylation in EC and MCF-7 cells (Razandi *et al.*, 2002) and EGFR-dependent PI3K/Akt1 phosphorylation in MCF-7 cells (Stoica *et al.*, 2003), the former by a mechanism involving G-protein activation (Razandi *et al.*, 2002). On the other hand, EGF can stimulate, within minutes, E_2-independent ERα activation via both Ras/MAPK (Bunone *et al.*, 1996) and PI3K/Ark (Martin *et al.*, 2000) signaling pathways, whereas IGF-I acutely stimulates E_2-independent ERα activation via PI3K/Ark (Aronica and Katzenellenbogen, 1993; Martin *et al.*, 2000), resulting in an increase in the number of receptors for progesterone (Martin *et al.*, 2000) and IGF (Lee *et al.*, 1999) in MCF-7 cells. Therefore, gene transcription induced by E_2-ERα complex acting alone or interacting with growth factors-growth factor receptors, emanates from rapid and specific

signaling, integrating cell surface and nuclear actions (Morey *et al.*, 1998; Watters and Dorsa, 1998; Watters *et al.*, 2000; Jager *et al.*, 2001; Levin, 2001; Pedram *et al.*, 2002).

Biological Actions of the Pure ER Antagonist ICI 182,780. The agonistic or antagonistic effects of ER modulators on actions of E_2 have been interpreted as indicative of ER in the mediation of the actions of E_2. ICI 182,780 was shown to be a pure antagonist of E_2 actions in the NOS system in every experimental condition tested. ICI 182,780 completely inhibited production of NO induced by long-term treatment with the phytoestrogen α-zeralenol in rats (Altavilla *et al.*, 2001) and the E_2-induced eNOS expression in human endothelial cells (Hayashi *et al.*, 1995), transiently transfected COS-7 cells (Nuedling *et al.*, 2001), and isolated COS-7 membranes (Chambliss *et al.*, 2000), as well as both down- and up-regulation of ERs by E_2 in endothelial cells (Ihionkhan *et al.*, 2002). Likewise, ICI 182,780 also prevented the acute (2 or 20 min) eNOS activation induced by E_2 (Chen Z *et al.*, 1999; Goetz *et al.*, 1999; Kim *et al.*, 1999 Simoncini and Genazzani, 2000;Russell *et al.*, 2000; Hisamoto *et al.*, 2001a; Hisamoto *et al.*, 2001b; Chen *et al.*, 2003), raloxifene (Simoncini and Genazzani, 2000; Hisamoto *et al.*, 2001a) or E_2-BSA (Russell *et al.*, 2000), and prevented different events in the E_2-induced activation of eNOS, like blockade of ERα-Gαi interaction (Wyckoff *et al.*, 2001), Akt activation (Hisamoto *et al.*, 2001b; Simoncini *et al.*, 2003), ERK 1/2 phosphorylation (Russell *et al.*, 2000; Simoncini *et al.*, 2003; Chen *et al.*, 2003), cGMP production (Russell *et al.*, 2000), and the E_2-induced rise in intracellular Ca^{2+} (Goetz *et al.*, 1999). ICI 182,780 alone had no effect on protein expression (Nuedling *et al.*, 2001), or eNOS activation (Chen Z *et al.*, 1999; Goetz *et al.*, 1999).

ICI 182,780 has also been tested in signaling pathways not related with the NOS system, and in the vast majority of studies ICI 182,780 behaved as an antagonist of the acute-, middle-, and long-term actions of E_2. For example, ICI 182,780 prevented the acute E_2-induced activation of MAP kinase cascades (Migliaccio *et al.*, 1996; Razandi *et al.*, 1999; Singer *et al.*, 1999; Razandi *et al.*, 2000a; Watters *et al.*, 2000; Wade *et al.*, 2001; Razandi *et al.*, 2002; Song *et al.*, 2002; Pedram *et al.*, 2002; Santos *et al.*, 2002; Mize *et al.*, 2003), activation of tyrosine kinases (Migliaccio *et al.*, 1996; Song *et al.*, 2002), and PI3K (Stoica *et al.*, 2002), phosphorylation of transcription factors (Razandi *et al.*, 2000a; Santos *et al.*, 2002; Song *et al.*, 2002; Wade *et al.*, 2003), secretion of prostaglandins (Pedram *et al.*, 2002), rapid gene transcription (Jager *et al.*, 2001; Pedram *et al.*, 2002), and prevented the E_2-induced inhibition of p38α kinase phosphorylation (Razandi *et al.*, 2000a). ICI 182,780 also prevented middle-term (those occurring within several hours) actions of E_2, like activation of MAP kinase (Razandi *et al.*, 2000b), tyrosine kinase (Lee *et al.*, 1999), DNA synthesis (Ignar-Trowbridge *et al.*, 1992; Curtis *et al.*, 1996; Lee *et al.*, 1999), ERα reporter gene activation by E_2 in rat pituitary cells (Demay *et al.*, 2001), and endothelial cells (Ihionkhan *et al.*, 2002), and the decrease in angiotensin II-mediated ERK secretion of endothelin-1 induced by E_2 (Morey *et al.*, 1998). In addition, the long-term (days to weeks) stimulatory effects of E_2 on sexual receptivity, uterine growth, body weight (Wadde *et al.*, 1993a, b), and mRNA for progesterone receptor (Curtis *et al.*, 1996; Fitzpatrick *et al.*, 1999) were blocked by ICI 182,780 (Fitzpatrick *et al.*, 1999). Therefore, it seems the antagonistic action of ICI 182,780 is due to occupancy of ER. Indeed, ICI 182,780 blocked *in vivo* binding of

[3H]estradiol in uterus, pituitary, and adipose tissue but not in the preoptic area of the hypothalamus; in contrast *in vitro*, ICI 182,780 competed for binding to neural estrogen receptors with an affinity comparable with that for E_2. These data suggest that this compound does not cross the blood-brain barrier (Wade *et al.*, 1993a). However, in neural tissue ICI 182,780 appears to have agonistic properties. In a neuroblastoma cell line, gene expression induced by E_2 was mimicked by ICI 182,780 and tamoxifen (Watters and Dorsa, 1998). In immature cerebellar neurons from rat ICI 182,780, like E_2, rapidly induced phosphorylation of ERK 1/2 (Wong *et al.*, 2003). Caution in interpreting these results is warranted because in both studies ICI 182,780 and tamoxifen were given at a dose 1000 times higher than E_2. Normally, they are administered at the same or a 10 times higher dose. Interestingly, in the last study 17α-E_2 also activated ERK phosphorylation, and the action of E_2 and ICI 182,780 were only seen in a discrete subpopulation of cells. In addition, although ICI 182,780 prevented E_2-induced IP3 production, E_2-induced ERK phosphorylation, and E_2-promoted ER-G-proteins interaction in CHO cells, ICI 182,780 did not prevented E_2-induced adenylate cyclase activation (Razandi *et al.*, 1999).

Biological Actions of Agonistic/Antagonistic ER Modulators. In the NOS system, tamoxifen acted as an antagonist whereas raloxifene acted as an agonist of E_2 actions. The long-term effect of E_2 on vasculature protection (Nechmad *et al.*, 1998) and the E_2-induced eNOS expression in human endothelial cells (Hayashi *et al.*, 1995) was decreased by tamoxifen. Tamoxifen also prevented the acute E_2-induced activation of eNOS in endothelial cells (Chen Z *et al.*, 1999), the E_2- or E_2-BSA-induced release of NO

in median eminence fragments (Prevot et al., 1999), and alone had no effect on any, genomic or nongenomic actions (Bourassa *et al.*, 1996; Chen Z *et al.*, 1999). As mentioned, raloxifene like E_2, activated eNOS within 10 min (Simoncini and Genazzani, 2000; Hisamoto *et al.*, 2001a) and the eNOS activation was dependent on Akt and ERK 1/2 phosphorylation in endothelial cells (Hisamoto *et al.*, 2001a). Although exceptions may exist, tamoxifen and 4-OH-tamoxifen behave as antagonists of acute actions of E_2 on several other signaling pathways. For example, the acute activation of MAP kinase (Singer *et al.*, 1996) and PI3K (Stoica *et al.*, 2002) by E_2, was prevented by tamoxifen and 4-OH-tamoxifen. Likewise, the anti-apoptotic action of E_2 was antagonized by these two compounds in human breast cancer cells, a mechanism mediated by activation of JNK1 and a cascade of intracellular cysteine proteases known as caspases, resulting in apoptosis (Mandlekar *et al.*, 2000a; Mandlekar *et al.*, 2000b; Mandlekar and Kong, 2001). However, when administered chronically the agonistic or antagonistic character of tamoxifen, 4-OH-tamoxifen, and raloxifene vary in a tissue-dependent manner. In ovine pituitary cells the increase GnRH receptors number induced by E_2 was blocked by tamoxifen (Gregg *et al.*, 1990). In a rat hypothalamic cell line, tamoxifen and raloxifene prevented E_2-induced expression of mRNA for progesterone receptors (Fitzpatrick *et al.*, 1999) and both blocked E_2-induced lordosis behavior in rats (Meisel *et al.*, 1987). However, tamoxifen alone did not increase the number of GnRH receptors (Gregg *et al.*, 1990). Likewise, tamoxifen or raloxifen did not affect expression of mRNA for progesterone receptor (Fitzpatrick *et al.*, 1999) or the binding of AP-1 in rat hypothalamic and pituitary tissues (Zhu and Pfaff, 1998). On the other hand, tamoxifen

and raloxifene also act as estrogen agonists in bone and other tissues (MacGregor *et al.*, 1998). Tamoxifen mimicked the long-term effects of E_2 on uterine growth and body weight, but prevented sexual receptivity; tamoxifen however, acted as antagonist when given together with E_2 (Wadde *et al.*, 1993a, c) which may reflect the lower RBA and REP of tamoxifen *vs.* E_2. An agonistic action of raloxifene has also been reported in ovariectomized rats (Pinilla *et al.*, 2001). In that study raloxifene treatment for 5 days decreased the pulsatile nature of the post-ovariectomy hypersecretion of LH and increased prolactin secretion.

Alternatively, it has been proposed that agonistic actions of synthetic ER ligands can be mediated by transcriptional activation of genes normally suppressed by E_2. In one study tamoxifen, 4-OH-tamoxifen, and ICI 182,780 activated transcription of quinone reductase gene, a gene whose transcription is normally suppressed by E_2 (Montano and Katzenellenbogen, 1997). Although ICI 182,780 and tamoxifen are not considered to bind preferentially to any ER subtype, it has been reported that the activation of MAPK by E_2 in ER-negative Rat-2 fibroblasts transfected with ERα plasmid was partially and completely blocked by tamoxifen and ICI 182,780, respectively; in contrast E_2-induced MAPK activation was unaffected by ICI 182,780 and actually increased by tamoxifen in cells transfected with ERβ plasmid (Wade *et al.*, 2001). No other reports have confirmed a selective action of ICI 182,780 or tamoxifen on ER subtypes.

Biological Actions of E_2-BSA. E_2-BSA is considered a membrane impermeable compound and is widely used to discriminate between genomic and nongenomic events, to provide evidence for signaling via the plasma membrane, and to show plasma

membrane binding sites for E_2. E_2-BSA mimicked the acute (15-min) effects of E_2 on cGMP production (Russell *et al.*, 2000), Akt (Hisamoto *et al.*, 2001b) and ERK 1/2 phosphorylation (Russell *et al.*, 2000; Chen *et al.*, 2003), and the subsequent activation of eNOS in endothelial cells (Kim *et al.*, 1999; Hisamoto *et al.*, 2001b; Chen *et al.*, 2003). Likewise, E_2-BSA rapidly released NO from median eminence fragments, and its effects were prevented by tamoxifen (Prevot *et al.*, 1999). E_2-BSA also mimicked the acute actions of E_2 on phosphorylation of several MAP kinases (Razandi *et al.*, 1999; Razandi *et al.*, 2000a; Wade *et al.*, 2001; Santos *et al.*, 2002; Wong *et al.*, 2003) and these effects were prevented by ICI 182,780 (Razandi *et al.*, 2000a; Wade *et al.*, 2001; Santos *et al.*, 2002). In addition, BSA by itself does not affect protein kinase signaling (Morey *et al.*, 1997; Watters *et al.*, 1997). Furthermore, and distinct from E_2, E_2-BSA did not stimulate reporter activity in tumoral and non-tumoral cell lines transfected with an ERE-luciferase reporter construct (Watters *et al.*, 1997; Razandi *et al.*, 1999; Razandi *et al.*, 2000b; Wade *et al.*, 2001). The lack of reporter activity has been evaluated up to 8 h after treatment with E_2-BSA (Razandi *et al.*, 2000b; Wade *et al.*, 2001) and it has been interpreted as evidence that E_2-BSA does not enter to the cell to bind to nuclear ER or dissociate into E_2 and BSA components. In addition, plasma membrane binding sites for E_2 have been detected in EC (Kim *et al.*, 1999; Russell *et al.*, 2000; Chen *et al.*, 2003) and rat pituitary tumor cell line GH_3/B6 (Pappas *et al.*, 1995) by using E_2-BSA conjugated to fluorescein isothiocyanate (FITC). In both cell types plasma membrane binding sites for E_2 were confirmed by labeled antibodies against ER (Pappas *et al.*, 1995; Russell *et al.*, 2000). In GH_3/B6 cells, the rapid release of prolactin induced by E_2 was mimicked by antibodies to

ERα, further supporting an action of E_2 on the plasma membrane (Pappas *et al.*, 1994; Norfleet *et al.*, 2000). Similarly, labeling studies in CHO cells transfected with ERα or ERβ showed plasma membrane binding sites for E_2-BSA-FITC, which was blocked by unlabeled E_2, ICI 182,780, and an antibody directed against the LBD of ERα, but not against the N terminus of ERα. Further, cell permeabilization resulted in dense labeling of the cell nucleus (Razandi *et al.*, 1999). Despite the successful use of E_2-BSA conjugate to mimic the *in vitro* acute action of E_2, it has been reported that E_2-BSA, but not E_2 activates ERK in SK-N-SH neuroblastoma cell line (Stevis *et al.*, 1999) and E_2-BSA failed to compete for binding of radioiodinated E_2 to ERs (Binder, 1984; De Goeij *et al.*, 1986; Stevis *et al.*, 1999), underscoring the need for additional cell impermeable analogs of E_2 for the characterization of membrane ERs (Stevis *et al.*, 1999).

Plasma Membrane ERs Subtypes as Mediators of E_2 Action. ERα (Kim *et al.*, 1999; Chambliss *et al.*, 2000) and ERβ (Chambliss *et al.*, 2002b) have been localized in isolated plasma membrane of endothelial cells in fraction highly enriched in caveolae (plasmalemmal signal-transducing domains) and are functionally coupled to eNOS. These results have been recapitulated in COS-7 and CHO cells cotransfected with either subtype of ER and eNOS (Chen Z *et al.*, 1999; Chambliss *et al.*, 2000; Hisamoto *et al.*, 2001b). Similarly, ERs have been localized in caveolar-like microdomains of plasma membrane preparations from cerebral cortex explants of developing mice and proved to mediate E_2-induced activation of ERK (Toran-Allerand *et al.*, 2002). In plasma membrane preparations from CHO cells transfected with either ER subtype (Razandi *et al.*, 1999; Razandi *et al.*, 2003) or from MCF-7 cells (Razandi *et al.*, 2000b), E_2 acutely

modulated several MAP kinases. These membrane preparations showed binding of labeled E_2, a nearly identical dissociation constant between membrane and nuclear fractions (Razandi *et al.*, 1999), and an interaction of caveolin 1 with ER was further corroborated (Razandi *et al.*, 2003). In addition, the rapid E_2-induced activation of protein kinases did not occur in ER negative cells; however, protein kinases in the same cells transfected with cDNA clones encoding ERs become responsive to E_2 (Migliaccio *et al.*, 1996; Razandi *et al.*, 1999; Wade *et al.*, 2001; Stoica *et al.*, 2002; Mize *et al.*, 2003 Wade *et al.*, 2003). Similarly, phosphorylation of protein kinase by E_2 occurs only after ERs are detected in the mature adipocyte (Santos *et al.*, 2002). From these studies, it appears that both ERα and ERβ mediate eNOS activation by E_2 at the level of the plasma membrane in endothelial cells. Similarly, in ER-negative Rat-2 fibroblasts (Wade *et al.*, 2001), the hippocampal-derived cell line HT22 (Mize *et al.*, 2003 Wade *et al.*, 2003), neonatal cardiomyocytes (Jager *et al.*, 2001), and CHO cells (Razandi *et al.*, 1999) transfected with cDNA clones encoding either ERα or ERβ, E_2 rapidly induced phosphorylation of MAPK. ER subtypes, however, may differentially modulate the activity of distinct MAP kinases in other cells types. For example, E_2 stimulated JNK activity in ERβ-expressing CHO cells, but it was inhibited in ERα-expressing CHO cells (Razandi *et al.*, 1999). Similarly, in MCF-7 cells, which express almost exclusively ERα (Register *et al.*, 1998), E_2 activated ERK (Razandi *et al.*, 2000a; Song *et al.*, 2002) but suppressed JNK phosphorylation (Razandi *et al.*, 2000a). The importance of ERα in the mediation of hormone actions has been underscored by the use of ERα knock out (ERKO) mice. As mentioned above, activation of ER by EGF-EGFR complexes increased mRNA for

progesterone receptors in mouse uterus; however, this response was absent in uterus from ERKO mice (Curtis *et al.*, 1996). The ERKO mice has been used successfully not only to gain insight in to the importance of ERα as mediator of E_2 actions but also to provide evidence suggesting a novel membrane ER mediating E_2-induced ERK phosphorylation in neural tissue during development. In this regard, Toran-Allerand and co-workers (1999, 2000, and 2002) reported that E_2 induced activation of the Src/Raf/ERK 1/2 cascade independently of the classic ERs in cerebral cortex explants of developing mice and ERKO mice. In these studies ICI 182,780 prevented ERK phosphorylation by E_2 in the wild type but not in the ERKO mice, whereas the selective ERα agonists 16α-iodo-17β-estradiol and PPT or the selective ERβ agonists genistein and coumestrol did not stimulate phosphorylation of ERK in ERKO mice. Moreover, detection of a protein immunoreactive for the LBD of ERα but not ERβ having a MW distinct from the nuclear ER subtypes located in caveolar-like microdomains of plasma membrane preparations, further support the existence of a novel membrane ER. In the wild type mice, 17α-estradiol, which has a 100-fold lower affinity for ERα than E_2 (Hajek *et al.*, 1997), elicited a stronger ERK activation than E_2, but in ERKO mice both 17α-estradiol and E_2 each activated ERK at pM concentrations. In addition, in wild type mice 16α-iodo-17β-estradiol and PPT inhibited ERK phosphorylation. This has been interpreted as evidence that in cerebral cortex of developing mice, E_2 prevents ERK phosphorylation via ERα. (Singh *et al.*, 1999; Singh *et al.*, 2000; Toran-Allerand, 2000; Nethrapalli *et al.*, 2001; Toran-Allerand *et al.*, 2002).

Biological Actions of ER Selective Agonists and an ER Selective Antagonist. The

ERα and ERβ selective agonists, PPT and DPN, respectively, rapidly induced (within 5 min) relaxation of pre-contracted isolated rat mesenteric arteries when given at pharmacological concentrations. Compared to the response induced by E_2, PPT had a significantly greater vasodilatory effect, while DPN had a significantly lesser effect. Thus, both ERα and ERβ can acutely relax isolated mesenteric arteries (Montgomery *et al.*, 2003). A role of ERβ in vascular protection and vasodilation induced by E_2 has been provided by the use of genistein, a less selective ERβ agonist than DPN (Barkhem *et al.*, 1998) and R,R-THC an ERβ selective antagonist (Sun *et al.*, 1999). Genistein caused a direct and rapid NO-dependent vasodilation in arteries of rats (Karamsetty *et al.*, 2001) and humans (Walker *et al.*, 2001), and it was more effective than E_2 in providing vascular protection, presumably mediated through ERβ, than in causing uterine stimulation, presumably mediated through ERα (Savolainen *et al.*, 1999). R, R-THC completely inhibited the eNOS up-regulation by E_2 in neonatal rat cardiac myocytes, indicating a primary role for the ERβ subtype (Nuedling *et al.*, 2001). These results agree with previous reports using cells transfected with cDNA encoding for ERα and ERβ. The biological action of PPT has also been characterized *in vivo*, and like E_2, PPT stimulated uterine weight gain, prevented loss of bone density, reduced plasma cholesterol levels after ovariectomy, and increased mRNA for progesterone receptors in hypothalamus, providing evidence for a role of ERα alone in the mediation of these effects (Harris *et al.*, 2002). Recently, the ability of a new ERβ selective agonist (ERB-041) to mimic E_2 action was evaluated (Harris *et al.*, 2003). The authors concluded that ERβ does not mediate the bone-sparing activity of estrogen on the rat skeleton and that it does not affect ovulation

or ovariectomy-induced weight gain. In addition, these compounds are non-uterotrophic and non-mammotrophic. However, ERβ may be an important modulater of the immune response.

Biological Actions of 17α-E_2 and Non-estrogenic Steroid Hormones on E_2-Induced Actions. The transcriptionally inactive stereoisomer 17α-E_2 initially was used as a criterion of specificity of 17β-E_2 (E_2) actions. Most acute actions of E_2 were not mimicked by 17α-E_2. For example, 17α-E_2 had no effect on vasoprotection (Bourassa *et al.*, 1996), Akt phosphorylation, or subsequent activation of eNOS in endothelial cells (Chambliss *et al.*, 2000; Hisamoto *et al.*, 2001b), in isolated plasma membrane (Chambliss *et al.*, 2000), or median eminence fragments (Prevot et al., 1999). Similarly, 17α-E_2 did not active MAP kinases, PI3K, PKC (Watters *et al.*, 2000; Razandi *et al.*, 2000a; Santos *et al.*, 2002), or cAMP production (Aronica *et al.*, 1994). However, in Rat-2 fibroblasts (Wade *et al.*, 2001), rat immature cerebral neurons (Wong *et al.*, 2003), and mouse immature cerebral cortex of wild type and ERKO mice (Toran-Allerand *et al.*, 2002), 17α-E_2 did activate MAP kinase. As discussed above, it has been suggested that 17α-E_2 may activate MAP kinases by an ER distinct from α or β subtypes (Behl *et al.*, 1997; Toran-Allerand *et al.*, 2002). As expected testosterone, progesterone, or dexamethasone, were unable to mimic the acute actions of E_2 on the distinct signaling pathways including NOS (Goetz *et al.*, 1999; Prevot et al., 1999), several protein kinases (Singer *et al.*, 1996; Watters *et al.*, 2000; Razandi *et al.*, 2000a; Razandi *et al.*, 2000b; Santos *et al.*, 2002; Wong *et al.*, 2003), cAMP production (Aronica *et al.*, 1994) or AP-1 binding (Zhu and Pfaff, 1998).

G-Protein Coupled Receptor (GPCRs)/Ion Channel Systems Modulated by E_2.

GPCRs are the largest class of membrane-bound receptors that include those for biogenic amines, proteins, polypeptide hormones, and neurotransmitters (Gilman, 1987). G-proteins are heterotrimers of α-, β-, and γ-subunits ($G_{\alpha\beta\gamma}$) that dissociate into Gα and Gβγ upon GPCR stimulation. After activation G_α and/or $G_{\beta\gamma}$ modulate the activity of downstream effector molecules (Gilman, 1987; Spiegel *et al.*, 1992) like PLC (Levitzki , 1987; Katz *et al.*, 1992); PKA (Daaka *et al.*, 1997), MAP kinases (Biensen *et al.*, 1996), PLA2 (Clark *et al.*, 1991), PI3K (Traynor-Kaplan, 1989), PLD (Thompson *et* al., 1991), NOS (Chambliss and Shaul, 2002a), and calcium (Birnbaumer *et al.*, 1991; Einhorn and Oxford, 1993) and potassium channels (Einhorn and Oxford, 1993). A role of G-proteins has been identified using non-hydrolyzable GTP analogs, like GDP-β-S and GTP-γ-S, which compete with intracellular GTP for the binding site of G-proteins on the α subunit. GDP-β-S locks GTPase in an inactive state, and GTP-γ-S activates G-proteins irreversibly (Gilman, 1987; Hepler and Gilman, 1992). The identity of the G-proteins has been determined by their sensitivity to cholera or pertussis toxins and by immunoblots using G-protein antibodies. This section discusses the potential role of G-proteins in the mediation of the rapid effects induced by E_2 on a variety of effector systems. However, it is important to mention that chronic treatment with E_2 has a significant and specific effect on G-protein levels in pituitary (Bouvier *et al.*, 1991; Livingstone *et al.*, 1998) and may promote a functional uncoupling of specific GPCRs with their effector system (Nunemura *et al.*, 1989), perhaps by stabilizing the association of the heterotrimeric G-protein subunits (Maus *et al.*, 1990). This may be the case for GPCRs like GnRH receptor

and D2 dopamine receptor, where chronic treatment with E_2 decreased inositol phospate (IP) breakdown induced by GnRH (McAldre *et al.*, 1992) and suppressed D2-dopamine-induced inhibition of adenylate cyclase (Maus et al., 1989). Evidence for a role of G-proteins in the mediation of acute actions of E_2 has been reported in osteoblasts (Lieberherr *et al.*, 1993; Le Malley *et al.*, 1997), macrophages (Benten, *et al.*, 2001), diverse neuronal cells (Mermelstein *et al.*, 1996; Caldwell *et al.*, 1999), and endothelial cells (Wyckoff *et al.*, 2001). The E_2-induced stimulation of eNOS observed in intact endothelial cells (Wyckoff *et al.*, 2001) and in COS-7 cells transfected with eNOS and ERα (Chen Z *et al.*, 1999; Wyckoff *et al.*, 2001) was prevented by pertussis toxin or by incubation of membrane endothelial preparations with GDPβS (Wyckoff *et al.*, 2001). In addition, overexpression of $G_{i1\alpha}$ augmented E_2-induced eNOS stimulation; whereas overexpression of (RGS)4, a regulator of G-protein which accelerates the GTPase activity (Huang *et al.*, 1997), attenuated the E_2-induced eNOS stimulation (Wyckoff *et al.*, 2001).

E_2 has a wide spread action on the modulation of calcium currents. In cells like rat osteoblasts (Lieberherr *et al.*, 1993; Fiorelli *et al.*, 1996; Le Mellay *et al.*, 1997), the murine macrophage cell lines IC-21 and RAW (Benten *et al.*, 2001; Guo *et al.*, 2002), and granulosa cells (Morley *et al.*, 1992), E_2 increased $[Ca^{2+}]i$ from intracellular stores, extracellular milieu, or both. Pertussis toxin prevented the increase in $[Ca^{2+}]i$ induced by E_2 (Lieberherr *et al.*, 1993; Le Mellay *et al.*, 1997). In osteoclast cells the E_2-induced increase in $[Ca^{2+}]i$ was mediated via PLC and IP3/DAG production (Lieberherr *et al.*, 1993; Le Mellay *et al.*, 1997) or associated with an increase in cAMP and cGMP (Fiorelli

et al., 1996) and plasma membrane binding sites for E_2 were detected using E_2-BSA-FITC (Fiorelli *et al.*, 1996). In addition, pertussis toxin also prevented E_2-mediated phosphorylation of MAPK in endothelial cells (Wyckoff *et al.*, 2001) and PLC activation and/or PI2/DAG production in osteoclast cells (Lieberherr *et al.*, 1993; Le Mellay *et al.*, 1997). In these cells types, E_2 action on $[Ca^{2+}]i$ was mimicked by E_2-BSA (Lieberherr *et al.*, 1993; Benten *et al.*, 2001; Guo *et al.*, 2002). Therefore, these data support G-protein activation as a primary step in the signaling pathway modulated by E_2. A potential interaction of plasma membrane ERs and G-proteins is suggested by the marked increase in coimmunoprecipitation of ERα and $G_{\alpha i}$ 20 min after treatment with E_2 in endothelial plasma membranes (Wyckoff *et al.*, 2001) and by a reduction in high affinity binding sites for E_2-[^{125}I-BSA] in membrane preparation from rat hypothalamus after preincubation of plasma membranes with cholera toxin or GTPγS (Caldwell *et al.*, 1999). The nature of such interactions is unknown and it has been speculated, that there are direct protein-protein interaction between ERs and GPCR, or a new intermediary protein may facilitate the ER-G-protein interaction (Wyckoff *et al.*, 2001). Additionally, it has been proposed that E_2 may bind to a classical GPCR that interacts directly with G-proteins. In support of this idea, in macrophages cell lines (Benten *et al.*, 2001; Guo *et al.*, 2002) and osteoblasts (Lieberherr *et al.*, 1993) tamoxifen, raloxifene, and/or ICI 182,780 did not prevent E_2-induced increase in $[Ca^{2+}]i$; whereas pertussis toxin prevented not only E_2-induced increase in $[Ca^{2+}]i$ (Lieberherr *et al.*, 1993; Benten *et al.*, 2001) but also inhibited internalization of E_2-BSA-FITC (Benten *et al.*, 2001). In hippocampal CA1 neurons a role for G-proteins in the action of E_2 on ion channels has been reported.

In these cells E_2 rapidly and reversibly potentiates kainate (glutamate) inward currents, and intracellular perfusion with GDPβS or GTPγS prevented or prolonged, respectively, potentiation of kainate-induced currents by E_2 (Gu and Moss, 1996; Gu and Moss, 1998). E_2-BSA, but not 17α-E_2, also potentiated kainate-induced currents, but only when applied simultaneously to either the extracellular plasma membrane and the cytosolic side of the membrane (Gu and Moss, 1998). In these studies, cAMP-PKA were implicated as mediators, however, a possible effect of GDPβS or GTPγS on cAMP-PKA was not evaluated begging the question about which event occurs first (G-protein or cAMP-PKA activation) in the signaling pathway that may phosphorylate kainate receptors. However, the E_2-induced potentiation of kainate currents was also present in hippocampal CA1 neurons from ERKO mouse, and ICI 182,780 did not prevent the action of E_2 in ERKO or wild type mice (Gu *et al.*, 1999). The investigators proposed existence of a distinct binding site for E_2, and suggest that a putative E_2 binding site appears to be coupled to AMPA/kainate receptor. Evidence for an interaction of E_2 with Maxi-K channels has been provided using HEK-293 cells. This cell line does not contain either nuclear-cytoplasmic receptors (Cerillo *et al.*, 1998) or an estrogen-binding site on their membranes (Wetzel *et al.*, 1998). In this study by using electrophysiology and ligand binding, a direct binding of E_2 and E_2-BSA to the regulatory β subunit of Maxi-K channels was demonstrated (Valverde *et al.*, 1999).

So far, evidence has been presented to support the conclusion that E_2 and E_2-BSA are capable of modulating ion channels by a mechanism that involves G-proteins. The fact that antiestrogenic compounds did not block the actions of E_2 on ion channels leads

to the suggestion that a distinct binding site for E_2 on the plasma membrane, perhaps a classical GPCR, may exist. However, the effect of E_2 and antiestrogens on ion channel activity is highly cell dependent. In rat neostriatal neurons (Mermelstein *et al.*, 1996) and vascular smooth muscle (Nakajima *et al.*, 1995; Ruehlmann *et al.*, 1998) E_2 inhibited, instead of stimulated, L-type Ca^{2+} currents, whereas other steroid hormones like testosterone, progesterone or 17α-estradiol did not affect Ca^{2+} currents in vascular smooth muscle (Nakajima *et al.*, 1995; Mermelstein *et al.*, 1996). In neostriatal neurons E_2, E_2-BSA, and to a lesser extent tamoxifen, inhibited Ca^{2+} currents and this effect was prevented by GTPγS (Mermelstein *et al.*, 1996). Similarly, in vascular smooth muscle ICI 182,780 inhibited L-type Ca^{2+} channels (Ruehlmann *et al.*, 1998). The fact that the E_2-induced inhibition of Ca^{2+} currents was mimicked by anti estrogenic compounds may suggest a role for the classic ERs in the mediation of this inhibitory action by E_2. The differential regulation by E_2 on the putative same ion channel (L-type Ca^{2+} channels) may be explained by its influence on more than one ion channel in the same cell. For example, it is known that relaxation of vascular smooth muscle occurs by stimulation of Maxi-K channels, inhibition of L-type Ca^{2+} channels, or both (Ruehlmann *et al.*, 1998). In agreement with that, in anterior pituitary cells, dopamine, acting on D2 receptors, concomitantly reduces calcium currents and increases potassium currents, known to inhibit prolactin release (Lledo *et al.*, 1992). These dopamine effects require GTP, are prevented by pertussis toxin or GDPβS, are potentiated by GTPγS, and apparently are mediated by $G_{o\alpha}$ (Lledo *et al.*, 1990; Lledo *et al.*, 1992). In addition to the mechanisms discussed to explain the role of G-proteins in mediating acute actions of E_2 on ion

channels, Kelly and Wagner (1999) proposed that E_2-ER complex may activate signaling pathways, which in turn uncouple GPCRs, like μ-opioid receptors and $GABA_B$ receptors, from effector systems like ion channels. In β-endorphin neurons, E_2 decreased the opening of inward rectifying K^+ channels, resulting in a decrease in the potency of μ-opioid and $GABA_B$ agonists to inhibit β-endorphin neurons (Lagrange *et al.*, 1994; Lagrange et al., 1997; Weatherill *et al.*, 1998). Inhibition of PKA and PKC activation (Kelly *et al.*, 1999; Lagrange et al., 1997; Kelly and Wagner, 1999) and the antiestrogen ICI-164,384 (Lagrange et al., 1997; Weatherill *et al.*, 1998; Kelly *et al.*, 1999) prevented E_2-induced decrease of inward rectifying K^+ channels opening. If this model is correct, then the action of E_2 on inward rectifying K^+ channels must be cell specific because in GnRH neurons E_2, like μ-opioid agonists, induce hyperpolarization by opening inward rectifying K^+ channels (Lagrange *et al.*, 1995). Although the cell type-dependent differential regulation of K^+ channels by E_2 remains to be evaluated, it has been proposed that such a mechanism might mediate, in part, the negative feedback of E_2 on the hypothalamic-pituitary axis (Kelly and Wagner, 1999).

Summary. Many aspects of the positive feedback of E_2 on LH secretion, as well as the negative feedback of E_2 on FSH secretion have been documented; however the mechanisms underlying the early decrease in secretion of LH caused by E_2 remain unknown. *In vivo* studies indicate that the early decrease in secretion of LH occurs, at least in part, at level of the pituitary gland via impairing GnRH-induced release of LH. It has been proposed that the short time frame (within an hour) required for E_2 to decrease secretion of LH in OVX ewes is incompatible with the classic genomic mechanism of

steroid hormone action; however, the precise time needed for E_2 to acutely decrease secretion of LH remains to be established. E_2 conjugated to a carrier molecule like BSA has been used to evaluate the nongenomic effects of E_2 *in vitro*, but concerns about its stability and capacity to bind to ERs have underscored the need to design a stable conjugated form of E_2. It is thought that the acute actions of E_2 are mediated via binding to proteins located on the plasma membrane, and the blocking of E_2 actions by most synthetic ligands of ERs has been used as evidence for a role of ERs in the mediation of acute actions of E_2. The two more abundant subtypes of ERs (α and β) have been detected on the plasma membrane of E_2 target cells and their importance in the modulation of second messenger signals has been proven. Recently, a new generation of ER synthetic agonists has been developed to selectively activate ERα or ERβ. These promise to be important tools for identifying roles of specific ER subtypes in mediating actions of E_2.

Therefore, we decided to establish *in vitro*, that as suggested by studies *in vivo*, E_2 rapidly decrease the secretion of LH by impairing the GnRH-induced secretion of LH, and determine whether the commercially available E_2-BSA, as well as a novel synthesized E_2-conjugate are able to mimic the effect of E_2 on secretion of LH. We propose that ERs mediate the acute negative feedback of E_2 on secretion of LH and this effect is differentially regulated by ER subtype (α or β). Finally, we predict that, in OVX ewes, E_2 as well as conjugated forms of E_2, will rapidly decrease pulsatile secretion of LH; however, as expected from the postulated nongenomic nature of the acute suppression of LH, E_2 but not its conjugated forms will induce a pre-ovulatory like surge of LH and decrease the secretion of FSH.

CHAPTER II

SYNTHESIS AND PURIFICATION OF ESTRADIOL-PEPTIDE (E_2-PEP) CONJUGATE AND RELATIVE BINDING AFFINITY (RBA) OF CONJUGATED FORMS OF E_2

Introduction. The most important criterion for establishing a nongenomic effect of steroid hormones on cell function is that the response has to be induced rapidly, within seconds to minutes (Revelli *et al.*, 1998). Further substantiation of the nongenomic action by steroid hormones has been demonstrated by the induction of the same rapid effects using steroids coupled to high-molecular weight proteins that putatively do not cross the plasma membrane. The commercially available E_2-BSA conjugate has been used successfully to mimic the acute actions of E_2 on a variety of cellular functions, and when conjugated to FITC, to localize E_2 binding sites on the plasma membrane. However, concerns regarding its stability in solution, contamination with free E_2, and ability to bind to ERs have been raised (Binder, 1984; De Goeij *et al.*, 1986; Stevis *et al.*, 1999) underscoring the need to design stable E_2 conjugates for the study and characterization of membrane-initiated actions. These concerns may explain why no attempts have been reported to evaluate the ability of E_2-BSA to mimic the acute actions of E_2 in an *in vivo* model. Some desirable characteristics of a conjugated form of E_2 are: 1) an E_2/carrier ratio of 1:1, 2) free of unconjugated E_2, 3) binds ER with a similar binding affinity to E_2, and 4) suitable for radiolabeling. The purpose of this study was to design, synthesize, and evaluate the binding of a novel E_2 conjugate possessing these characteristics.

Materials and Methods

Conjugation of E_2 to an Amino Acid Sequence. Six-keto-17β-estradiol-6-carboxy methyl oxime (E_2-6-CMO; Steraloids Inc.) was conjugated to the amino group of serine of a 15 amino acid sequence (N-terminus-SGGEVVVDQPMERLY-C-terminus; PEP; Macromolecular Resources, Colorado State University). The use of E_2 conjugated on the carbon 6 to CMO will not interfere with the binding of antibodies or receptors to the hydroxyl radical at positions 3 and 17 in the E_2 molecule (Jensen, 1962; Noteboom and Gorski, 1965; Niswender *et al.*, 1975). The reactions were carried out at 10 °C, at pH 8, using N, N, dimethylformamide (DMF) as solvent as described by (Erlanger *et al.*, 1959). Based on molecular weight (MW) a 2:1 ratio was used for E_2-6-CMO/PEP, whereas a 1:1 ratio was used for E_2-6-CMO/Tri-N-butylamine and E_2-6-CMO/isobutyl chloroformate. Reactions were performed as follows; 4-6 mmoles of E_2-6-CMO dissolved in 0.5 ml of DMF were allowed to react for 10 min with Tri-N-butylamine; then isobutyl chloroformate was added and the reaction continued for 20 min. Finally, 2-3 mmoles of PEP were dissolved in DMF and the reaction was continued for an additional 60 min before termination by the addition of ~10 μl of 0.1 M acetic acid.

Purification of E_2-PEP Conjugate. Conjugation reactions were added to a 50 ml LH20-sephadex column, using methanol as solvent. Twenty-five fractions (2 ml each) were collected. Presence of reactants was determined by optical densitometry at a wavelength of 280 nm. Identification of the reactants in the eluate was determined based on their MW by Mass Spectrum Analysis (MSA; Macromolecular Resources, Colorado State University) using two different procedures; Matrix Assisted Laser Desorption Ionization (MALDI) Time of Flight (TOF) for compounds with a MW over 1000 (free

PEP and E_2-PEP conjugate), and electro spray mass spectrometry (ES) for compounds with a MW below 500 (free E_2-6-CMO). Conjugation efficiency was calculated based on the amount of unconjugated E_2-6-CMO after separation of reactants by chromatography. The weight of unconjugated E_2-6-CMO was estimated by plotting the absorbency values against those from known amounts of E_2-6-CMO.

E_2-BSA (~30 molecules of E_2 attached to each molecule of BSA) was purchased from Steraloids, Inc. (Newport, Rhode Island). To remove free E_2, about 20 mg of E_2-BSA were dissolved in 2ml of phosphate buffered saline (PBS) in a 16 x 150 mm glass tube. Five ml of diethylether were added and the contents of the tubes were vortexed for 1 minute and then frozen in dry-ice methanol. The organic fraction was poured into a 12 x 75 mm glass tube and evaporated under nitrogen in a warm heating block. Dried extracts were reconstituted in PBS containing 0.1% gel, incubated at 4 °C overnight, and vortexed before quantification of E_2 by radioimmunoassay. The extraction procedure was repeated four times for each sample and each extract was kept separate and assayed for estradiol (Korenman *et al.*, 1974).

Relative Binding Affinity (RBA) of E_2-BSA and E_2-PEP Conjugates. Relative binding affinity of conjugated forms of E_2 was determined in the cytosolic fraction of ovine uterus using [2,4,6,7,16,17-^{3}H]17-β-estradiol ([^{3}H] E_2, 141 Ci/mmol; Dupont NEN) as radiolabeled ligand, and serial dilutions of E_2, E_2-BSA or E_2-PEP as inhibitory competitors. Uterine tissue was weighed and homogenized in ice-cold TEDG buffer [10 mM Tris-HCl, 1.5 mM ethylenediamine-tetraacetic acid; 2 mM dithiothreitol, and 10% (v/v) glycerol; pH = 7.5] using a polytron homogenizer (1 g tissue in 10 ml TEDG). Homogenates were centrifuged at 30,000 x g for 60 min at 4 °C and the supernatant

(cytosol) was harvested. Tissue processing and receptor assay was carried out as previously reported by Amann *et al.*, (1986). Binding capacity of ER in the cytosols was determined by using an exchange assay. Duplicate 200 µl aliquots of cytosol were incubated with an equal volume of buffer containing 4 nM [^{3}H]-E_2, with or without a serial dilution of E_2, E_2-PEP or E_2-BSA. Because the variable number of E_2 molecules attached to BSA, the estimation of concentrations of E_2-BSA was based on the MW of BSA. Aliquots containing cytosol, 4 nM [^{3}H]-E_2, and a 100-fold excess E_2 was used to estimate non-specific binding. After incubation for 30 min at 4 °C, ice-cold dextran-coated charcoal was added, after 10 min samples were centrifuged at 3000 x g at 4 °C for 15 min and the amount of [^{3}H]-E_2 remaining in the supernatant (i.e. bound to receptor) was determined. RBA of conjugated forms of E_2 was calculated by dividing the molar concentration of E_2 needed to reduce specific [^{3}H]-E_2 binding by 50% by the molar concentration of E_2-BSA or E_2-PEP that reduced specific [^{3}H]-E_2 binding by 50% (RBA = IC_{50} E_2/IC_{50} Conjugate X 100). Binding affinity of E_2 was arbitrarily assigned a value of 100 % (Wakeling, 1987).

Results. Two major peaks were detected by optical densitometry in fractions collected from LH20-sephadex column chromatography of the reactants subjected to conjugation. A representative example is presented in Figure 2.1. Mass Spectrum Analysis revealed two main compounds in the fractions 9 to 11, one with 1679 MW corresponding to unconjugated PEP, and another one with 2020 MW corresponding to E_2-PEP conjugate. The main compound detected in fractions 13 to14 had a MW of 359 corresponding to unconjugated E_2-6-CMO. Conjugation efficiency in individual conjugations ranged from 20 to 100% (n = 10).

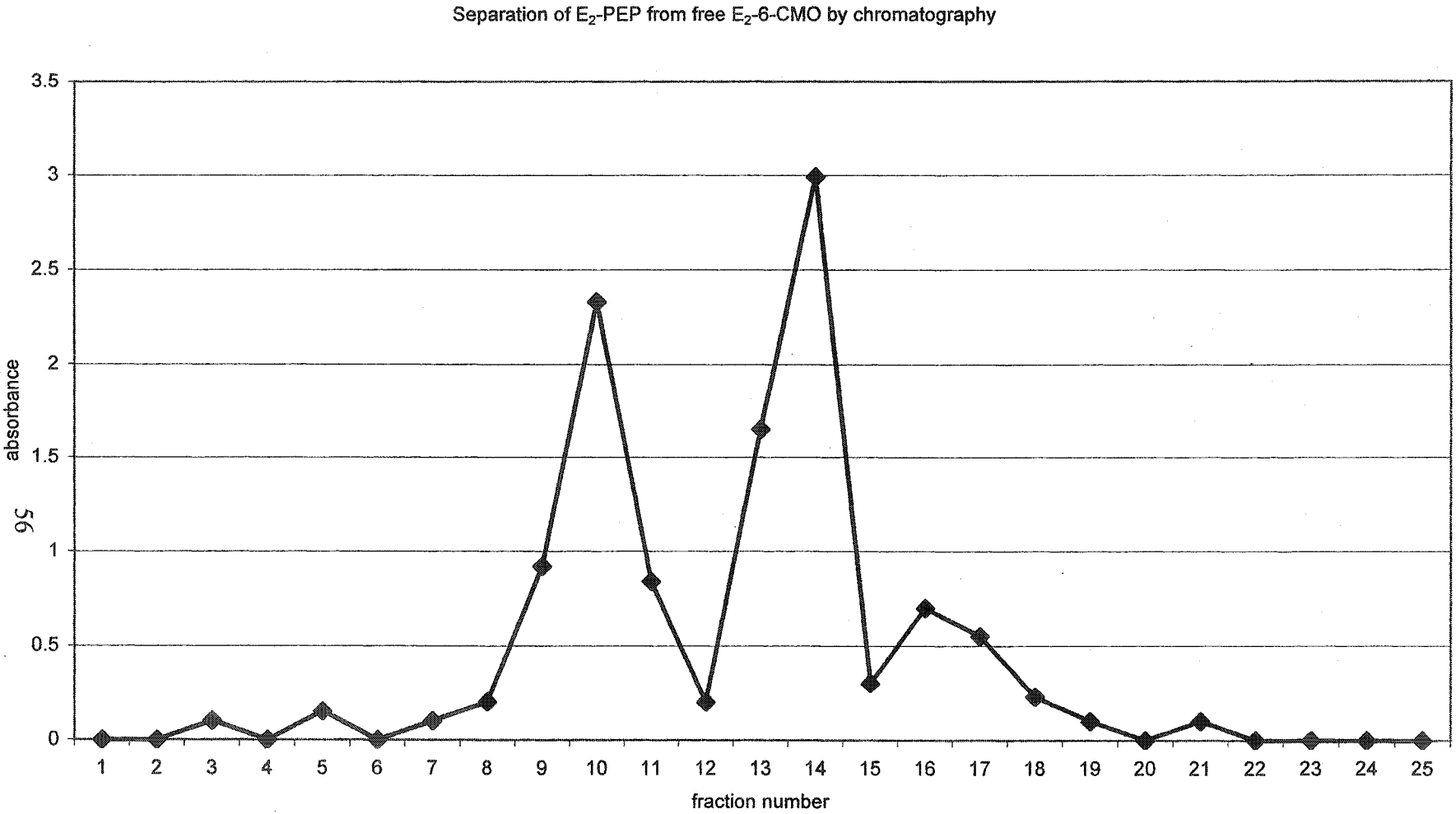


Figure 2.1. Optical densitometry detected 2 peaks from fractions collected after LH20-sephadex column chromatography. Mass Spectrum Analysis revealed two main compounds in the fractions 9 to 11, one with 1679 MW corresponding to free PEP, and another with 2020 MW corresponding to E_2-PEP. The main compound detected in fractions 13 to14, with 359 MW corresponded to free E_2-6-CMO

Extraction with diethyl ether removed 13.2% (364.6 ± 98.43 μg E_2; n = 6 extractions) of total expected E_2 (at a 30:1 ratio) present in 20 mg of E_2-BSA powder (Figure 2.2). The amount of free E_2 represents 1.8% of the weight of the conjugate. RBA for E_2-BSA and E_2-PEP were 9 and 89%, respectively compared to E_2 (Figure 2.3).

Discussion. E_2 was successfully conjugated to a 15 amino acid sequence through the amino group on the N-terminal amino acid. The E_2:PEP ratio was corroborated by MSA based on the MW of these compounds. Separation of conjugated from unconjugated E_2-6-CMO by LH20 column chromatography was further corroborated by MSA. Relative binding affinity of E_2-PEP revealed that the attachment of a 15 amino acid sequence to E_2 did not alter its ability to bind to uterine homogenates. Moreover, the presence of a tyrosine residue at the C-terminal position in the PEP will permit radioiodination of the conjugate, and therefore its characterization as an ER ligand may provide a better ligand to study the kinetics of binding to plasma membrane ERs. Therefore, this new E_2 conjugate has some characteristics that make it more desirable for use as a ligand to evaluate the nongenomic actions of E_2 than the commercially available E_2-BSA. The biological activity of E_2-PEP is addressed in subsequent chapters.

Steroid-BSA conjugates have been reported to contain unbound or adsorbed steroid in the range of 2-4% (Erlanger *et al.*, 1959) or 1% (Gaetjens and Pertschuk, 1980) of the weight of the conjugate. In the present study, 1.8% of the weight of the conjugate was removed as free E_2 from the E_2-BSA by solvent extraction. The amount of free E_2 remaining in the E_2-BSA conjugate after five extractions was considered insignificant. If E_2-BSA was administrated at the equimolar (~13 mg E_2-BSA) concentrations of E_2 used *in vivo* studies (i.e. ~50 μg; Reeves *et al.*, 1974; Gregg and Nett, 1989) and if the amount

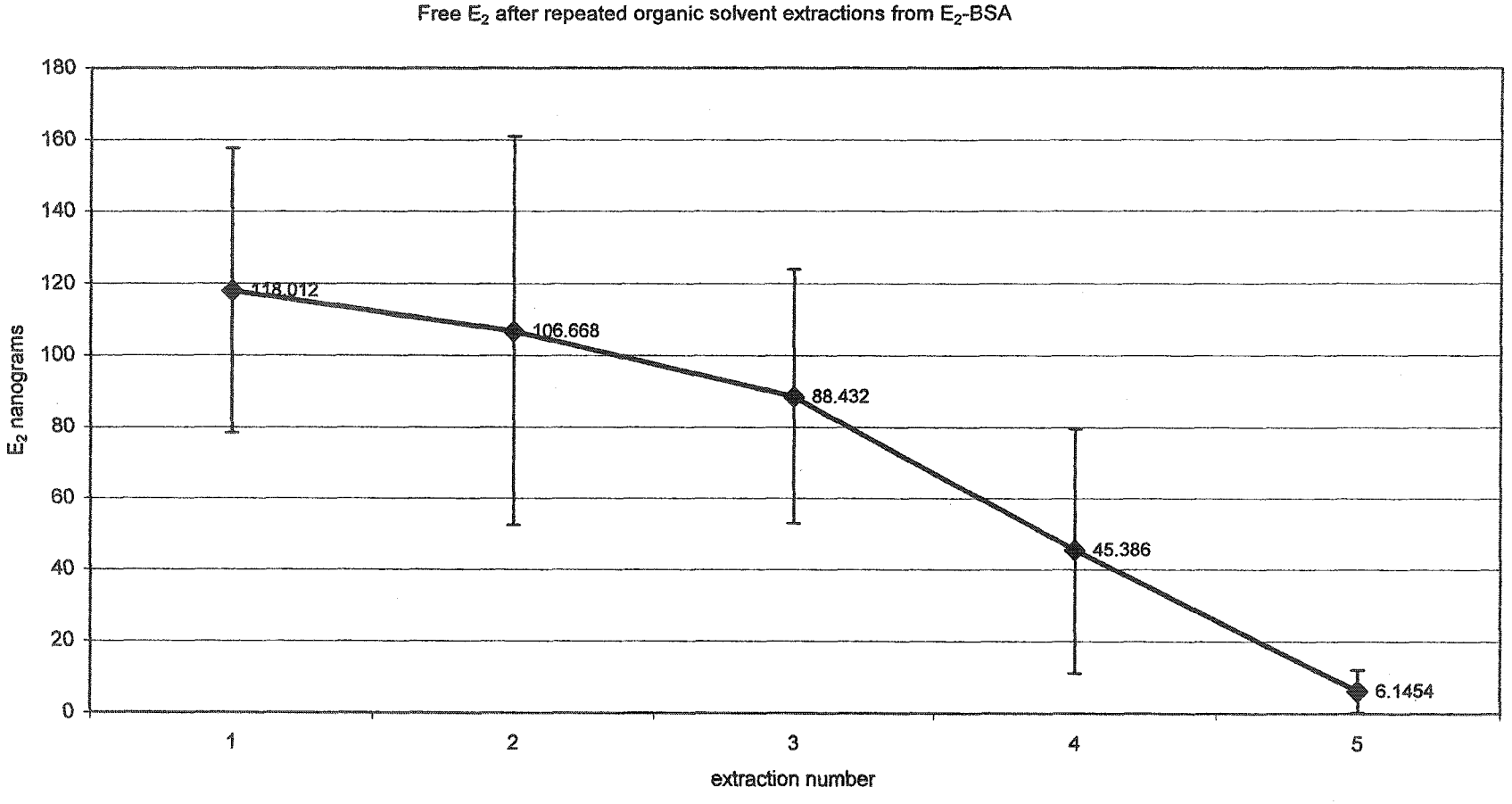


Figure 2.2. The organic solvent extraction procedure removed 13.2% (364.6 ± 98.43 µg E_2; n = 5 samples extracted) of total E_2 present in 20 mg of E_2-BSA powder, as determined by radioimmunoassay. Error bars represents standard deviation.

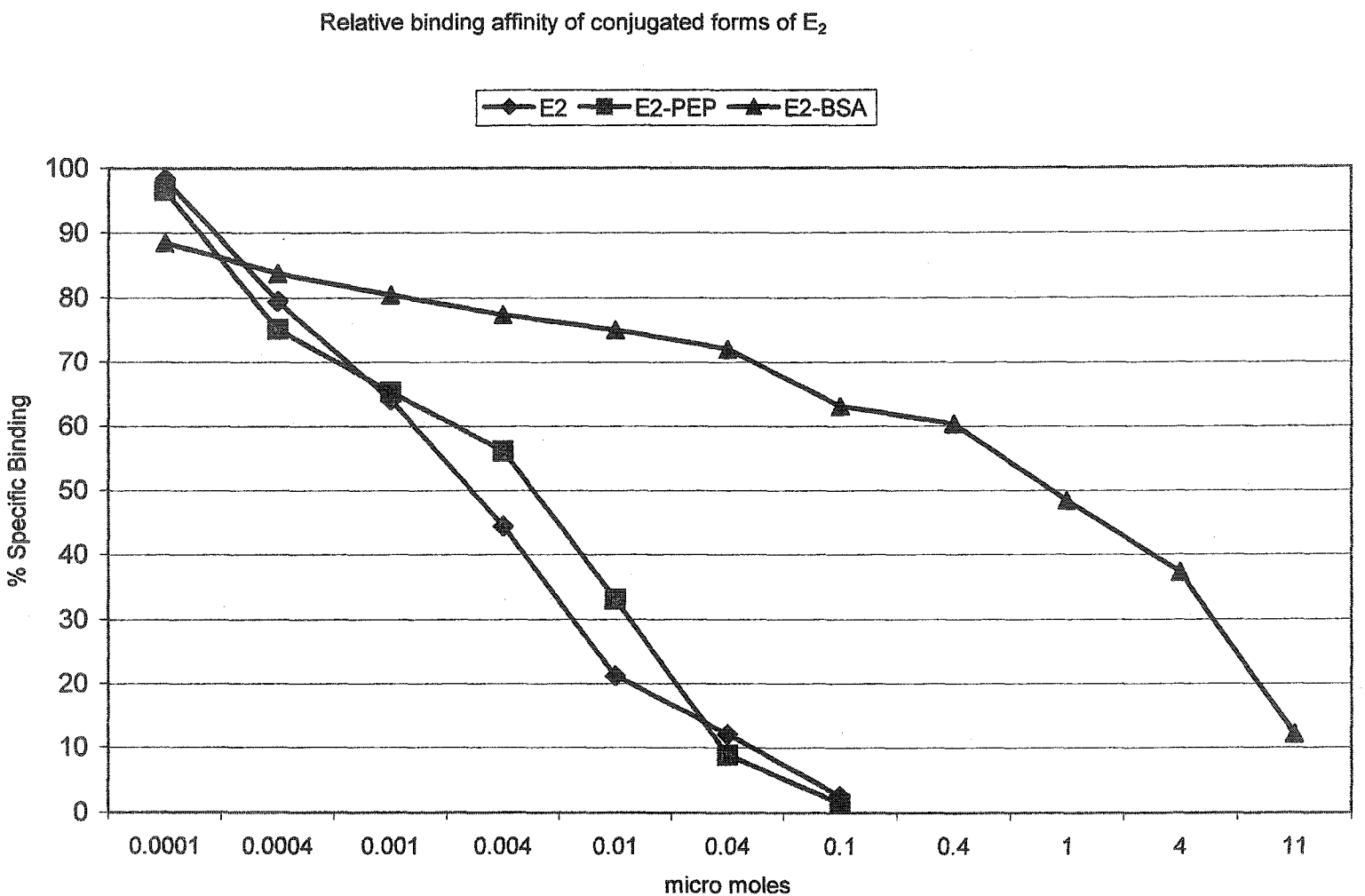


Figure 2.3. Relative binding affinity (RBA) of E_2-PEP and E_2-BSA compared to E_2. RBA= IC_{50} E_2/ IC_{50} Conjugate X 100. Data from a representative Replicate. Experiment was repeated twice using ovine uterine homogenate tissue at 1:2 dilution.

of free E_2 present in the conjugate even before the last extraction (~300 pg free E_2//mg E_2-BSA) were considered for this estimation, the expected increase in serum concentration, due to free E_2, would be in the order of femtograms per ml of serum. This is substantially below the concentration needed to elicit a physiological response.

Radioreceptor assay showed that E_2-BSA, like E_2, was able to bind to uterine homogenates. The ability of E_2-BSA to bind to E_2 binding sites has been demonstrated using radioiodinated E_2-BSA (E_2-^{125}I-BSA) in membrane preparations of OVX rat hypothalamus (Caldwell, *et al.*, 1999), rat olfactory bulb (Zheng *et al.*, 1996), and membrane E_2 binding proteins from rat hypothalamus immobilized on nitrocellulose membranes (Zheng and Ramirez, 1997). Binding sites for E_2-BSA-FITC have also been detected in membranes of MCF-7 cells (Berthois *et al.*, 1986). Similarly, radioreceptor assay using uterine cytosol has shown binding of E_2-BSA to E_2 binding sites (Binder, 1984; De Goeij *et al.*, 1986). In our study the RBA of E_2-BSA was 9%, which is similar to the RBA of 7.5% reported by others using uterine cytosol (De Goeij *et al.*, 1986); however, it has been reported that E_2-BSA is unstable in solution, especially when incubated with cytosol, resulting in a release of free immunoassayable E_2 from the conjugate (Binder, 1984; De Goeij *et al.*, 1986). In those studies, when RBA was determined immediately after removal of free E_2 by diethyl ether, charcoal adsorption, gel filtration, or dialysis, the RBA of E_2-BSA was undetectable or ~1.5% (Binder, 1984; De Goeij *et al.*, 1986; Stevis *et al.*, 1999). In our study, E_2-BSA was subjected to repeated diethyl ether extractions and stored for up to several months at –20 °C before evaluation of its RBA. If, as suggested in those studies, the binding of E_2-BSA to ERs is due almost completely to the presence of free E_2, and considering the polyvalent nature ~30 to 1

E_2:BSA ratio of the conjugate, the RBA expected from E_2-BSA containing free E_2 would be much higher than the 9% detected in this experiment. In agreement with this interpretation, *in vitro* studies indicate that E_2-BSA is able to mimic the acute actions of E_2 at the same or 10 times higher concentration than E_2 (Watters *et al.*, 1997; Razandi *et al.*, 1999; Kim *et al.*, 1999; Razandi *et al.*, 2000a; Hisamoto *et al.*, 2001b; Wade *et al.*, 2001; Santos *et al.*, 2002; Wong *et al.*, 2003; Chen *et al.*, 2003).

In conclusion, a novel E_2 conjugate, containing one molecule of E_2 by carrier molecule, suitable for radiolabeling, and free of unconjugated E_2, proved to bind to binding sites in uterus homogenates with a RBA similar than that of E_2. The commercially available E_2-BSA, relatively free of unconjugated E_2, also bound to binding sites in uterus homogenates with a RBA ~10 lower than that of E_2. Therefore, both E_2 conjugates promise to be biological active.

CHAPTER III

ACUTE NONGENOMIC ACTION OF E_2 AND TWO CONJUGATED FORMS OF E_2 ON THE INHIBITION OF GnRH-INDUCED SECRETION OF LH IN PRIMARY CULTURES OF OVINE PITUITARY CELLS

Introduction. As detailed in the review of literature, *in vivo* studies in ewes support the concept that E_2 decreases secretion of LH via a direct effect on the pituitary gland by interfering with the ability of GnRH to stimulate release of LH (Nett *et al.*, 1984; Clarke *et al.*, 1988; Gregg and Nett, 1989; Mercer, *et al.*, 1993; Molter-Gerard, *et al.*, 2000). Because of the short time-frame required for the decrease in serum concentrations of LH after treatment with E_2, Nett *et al.*, (1984) proposed that E_2 may suppress secretion of LH by a means different from the classic genomic mechanism.

In primary cultures of ovine pituitary cells, however, an acute suppression of GnRH-induced secretion of LH by E_2 has not been reported. Conjugated forms of E_2 have been widely used to mimic the acute, nongenomic, presumably membrane-initiated actions of E_2 (Razandi *et al.*, 1999; Kim *et al.*, 1999; Razandi *et al.*, 2000a; Hisamoto *et al.*, 2001b; Wade *et al.*, 2001; Santos *et al.*, 2002; Wong *et al.*, 2003; Chen *et al.*, 2003). Therefore, we used primary cultures of ovine pituitary cells from OVX ewes during the breeding season as the experimental model to evaluate: 1) the suppressive effect of E_2 on GnRH-induced secretion of LH; 2) the lag-time required for E_2 to effectively suppress LH secretion in response to a GnRH challenge; and 3) if conjugated forms of E_2 can mimic the negative feedback effect of E_2 on LH secretion, supporting a nongenomic

action as the mechanism underlying the acute suppression of LH secretion, and suggesting this effect of E_2 is mediated at level of the plasma membrane.

Materials and Methods

Preparation of Media and Stock Solutions. Dissociation medium consisted of 137 mM NaCl; 5 mM KCl; 25 mM n-2-hydroxyethyl piperazine ethane sulfonic acid (HEPES; United States Biochemical Corp., Cleveland, OH); pH 7.3, plus an enzymatic cocktail containing 1.0 mg/ml collagenase (type II), 1.0 mg/ml hyaluronidase (type V), and 0.02 mg/ml deoxyribonuclease. Enzymes were freshly prepared immediately prior to dissociation. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DME; Gibco, Lab.) supplemented with 10% OVX ewe serum, streptomycin sulfate (500 mg/ml), potassium penicillin G (313 mg/ml), and 2.2 g/L $NaHCO_3$. Dissociation and culture medium, as well as the enzymatic cocktail were sterilized by filtration through 0.2 μm millipore membranes (type GS, Millipore Corp.). Stock solutions of GnRH (in saline solution plus 0.1% BSA), E_2-BSA or E_2-PEP (in saline solution) were stored at –20 °C in small aliquots. E_2 was freshly dissolved in ethanol the day of pituitary treatment.

Dissociation and Incubation of Pituitary Cells. All procedures involving animals were approved by the Colorado State University Animal Care and Use Committee and complied with National Institute of Health (NIH) guidelines. Anterior pituitary glands were collected during breeding season, following anesthesia of ewes with sodium pentobarbital and exsanguination. Tissues were removed and immediately placed in ice cold dissociation medium. Anterior pituitary tissue from OVX ewes was dispersed as described by Adams et al. (1979a) with the omission of trypsin digestion. Briefly, tissue was sectioned into 0.5-mm-thick slices with a Stadie-Riggs tissue slicer and washed 5

times with dissociation medium without enzymes. Tissue was incubated in dissociation medium containing the enzymatic cocktail at 37 °C in a Dubnoff metabolic shaker for 90 min, and every 30 min the cell suspension was passed through a Pasteur pipette. After dissociation, the cell suspension was washed (400 x g; 4 min) 5 times with dissociation medium without enzymes, re-suspended in DMEM and plated at 2 x 10^5 cells per well in 24-well tissue culture plates (Corning Glass Works). Cells were incubated for 2 days at 37 °C under an atmosphere of 95% air: 5% CO_2. Cell viability was evaluated immediately after tissue dissociation and before administration of treatments by incubating the cells with 1% trypan blue for 3 to 4 minutes.

Radioimmunoassay. The amount of LH released from primary cultures of ovine pituitary cells was quantified by a double antibody radioimmunoassay (Niswender et al., 1969). The reference standard was NIH-OLH-S24. Triplicate standard curves were included in each assay and samples were analyzed in duplicate at 50 µl sample/tube. Ovine LH was radiolabeled with 1 mCi Na ^{125}I (Amersham Pharmaci Biotech: Buckinghamshire, England) by the chloramine T procedure (Niswender *et al.*, 1975). Briefly, 5 µg oLH (in 50 µl 0.5 M phosphate buffer) plus 1 mCi Na ^{125}I were allowed to react for 2 min with 5 µg (in 15 µl 0.05 M phosphate buffer) chloramine T. Reaction was stopped by adding 60 µg (30 µl 0.05 M phosphate buffer) sodium metabisulfite. Radioiodinated compounds were separated from free ^{125}I by passage of the reaction mixture through a 10 ml G-25-sephadex column. The column was eluted with PBS-gel, and 1 ml fractions were collected. The radioactive peak containing ^{125}I-oLH was detected in fractions 2-3.

Experimental Procedure. The first set of experiments was performed to evaluate the effect of E_2, E_2-BSA, and E_2-PEP on GnRH-induced release of LH from cultured pituitary cells. After 2 days incubation, anterior pituitary cells were washed twice with culture medium and treatments were applied in 1 ml of medium. Cells (2×10^5 cells per well; 4 wells per treatment, and 3 replicates (pituitaries) per experiment) were pre-incubated for 60 min with 0, 0.01, 0.1, 1, 10 or 100 nM E_2, E_2-BSA or E_2-PEP. After pre-incubation, cells were washed once with medium and incubated for 15 min with culture medium (negative control) or the previous treatment of E_2, E_2-BSA or E_2-PEP plus 2 nM GnRH. To prevent contamination with cells, culture medium was collected, centrifuged at 400 x g for 5 min, transferred (~90% of medium) to a 12 x 75 mm plastic culture tube, and stored at –20 °C for quantification of LH. In a preliminary study when 2nM GnRH was applied to 2×10^5 cells it induced a 10-fold increase in release of LH compared to the culture medium from untreated cells. When ethanol was used as solvent, its final concentration in the culture medium was never more than 0.1%, and the addition of 0.1% of ethanol to the GnRH treated cells did not affect the amount of LH released compared to GnRH treated cells. A second set of experiments was performed to evaluate the effect of pre-incubation time (15, 30 or 60 min) with 0 to 100 nM E_2, E_2-BSA or E_2-PEP on the amount of LH released during a subsequent 15 min GnRH challenge from cultured pituitary cells. With exception of the pre-incubation time, cells were processed and treated as described above. The experiments were run in two parts. In the first one, dispersed pituitary cells were pre-incubated for 30 or 60 min (control group); in the second part of this experiment, cells were incubated for 15 or 60 min (control group).

Statistical Analysis. Data were subjected to analysis of variance (ANOVA) using the general linear model of SAS (1987) in a completely randomized design. When a significant F-value occurred, means were separated using LSD adjusted by Tukey's procedure.

Results. The dissociation procedure yielded 75.4 ± 0.40 x 10^6 cells per pituitary (n=9). Viability of cultured pituitary cells, after tissue dissociation was always greater than 95% (96.8 ± 0.5%; n=9). After incubation for 2 day, cell viability was always greater than 80% (85.9 ± 1.1%; n=9). Coefficient of variation, intra- and inter-assay (n=9) for LH were 3% and 6%, respectively, and the minimum detectable dose of LH averaged 30 pg. Incubation of anterior pituitary cells with 2 nM GnRH for 15 min increased release of LH compared with untreated cells (53.11 ± 2.46 versus 3.3 ± 2.01 ng/ml; $p < 0.01$). Treatment with E_2 (Figure 3.1), E_2-BSA (Figure 3.2) or E_2-PEP (Figure 3.3) inhibited GnRH-induced release of LH in a dose-dependent manner. For E_2 treated cells, 0.1 nM E_2 decreased release of LH compared with GnRH treated cells but 10 nM E_2 was required to totally abolish GnRH-induced release of LH (Figure 3.1). Similarly, 0.1 nM of E_2-BSA (Figure 3.2) or E_2-PEP (Figure 3.3) decreased release of LH compared with GnRH treated cells and like with E_2, 10 nM of each conjugate was required to totally abolish GnRH-induced release of LH. Pre-incubation time (30 versus 60 min) did not alter the inhibitory effect of E_2, E_2-BSA or E_2-PEP on GnRH-induced release of LH. No significant interactions between incubation time and dosage (0.01, 0.1, 1, 10, and 100 nM; $p > 0.1$) or between treatment (E_2, E_2-BSA or E_2-PEP) and dosage ($p > 0.1$) were found; therefore, treatments were pooled by dosage and the interaction of treatment with

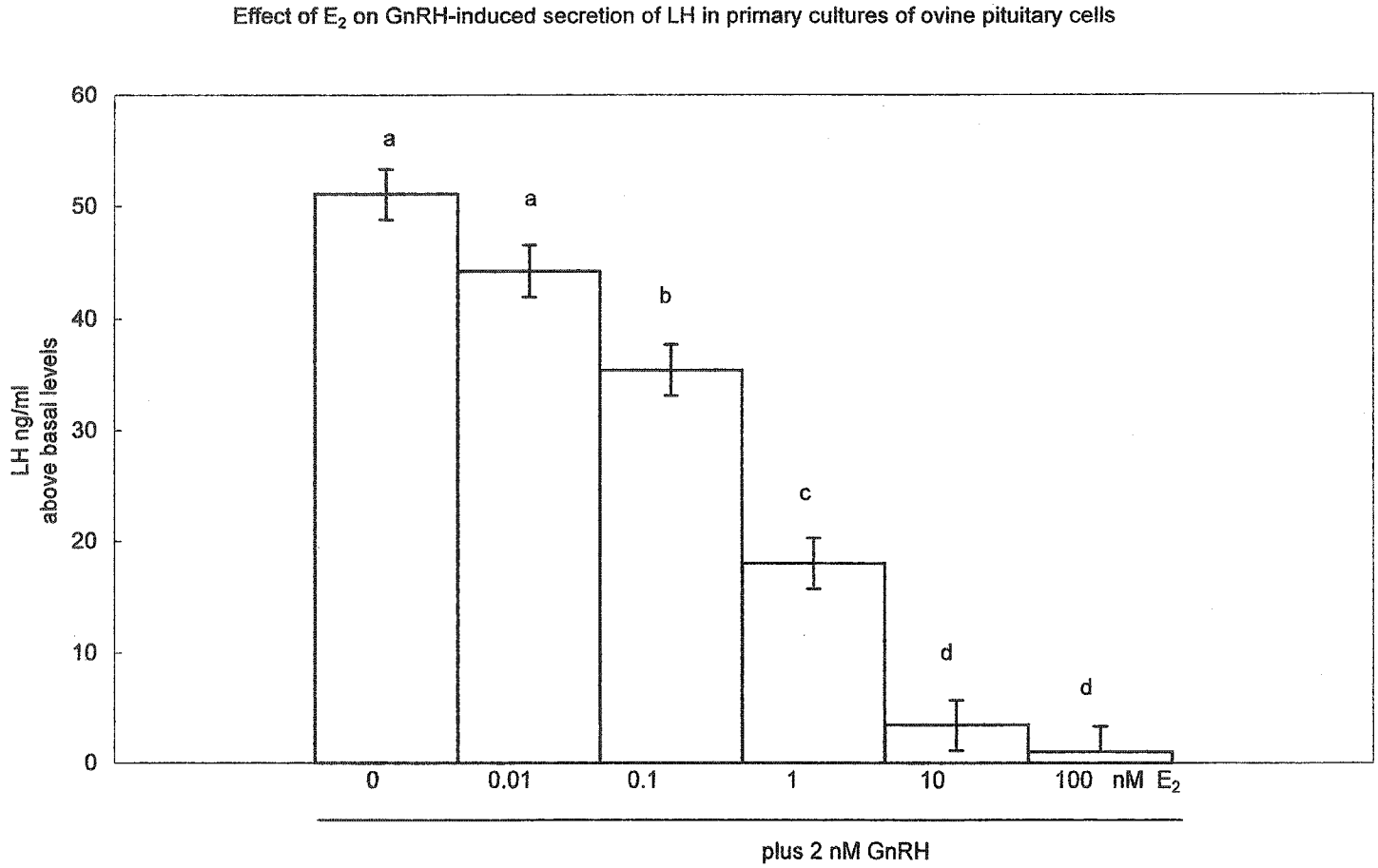


Figure 3.1. Cells (2×10^5 cells per well) were pre-incubated for 60 min with culture medium or E_2, washed and incubated for 15 min with the previous treatment plus 2 nM GnRH. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

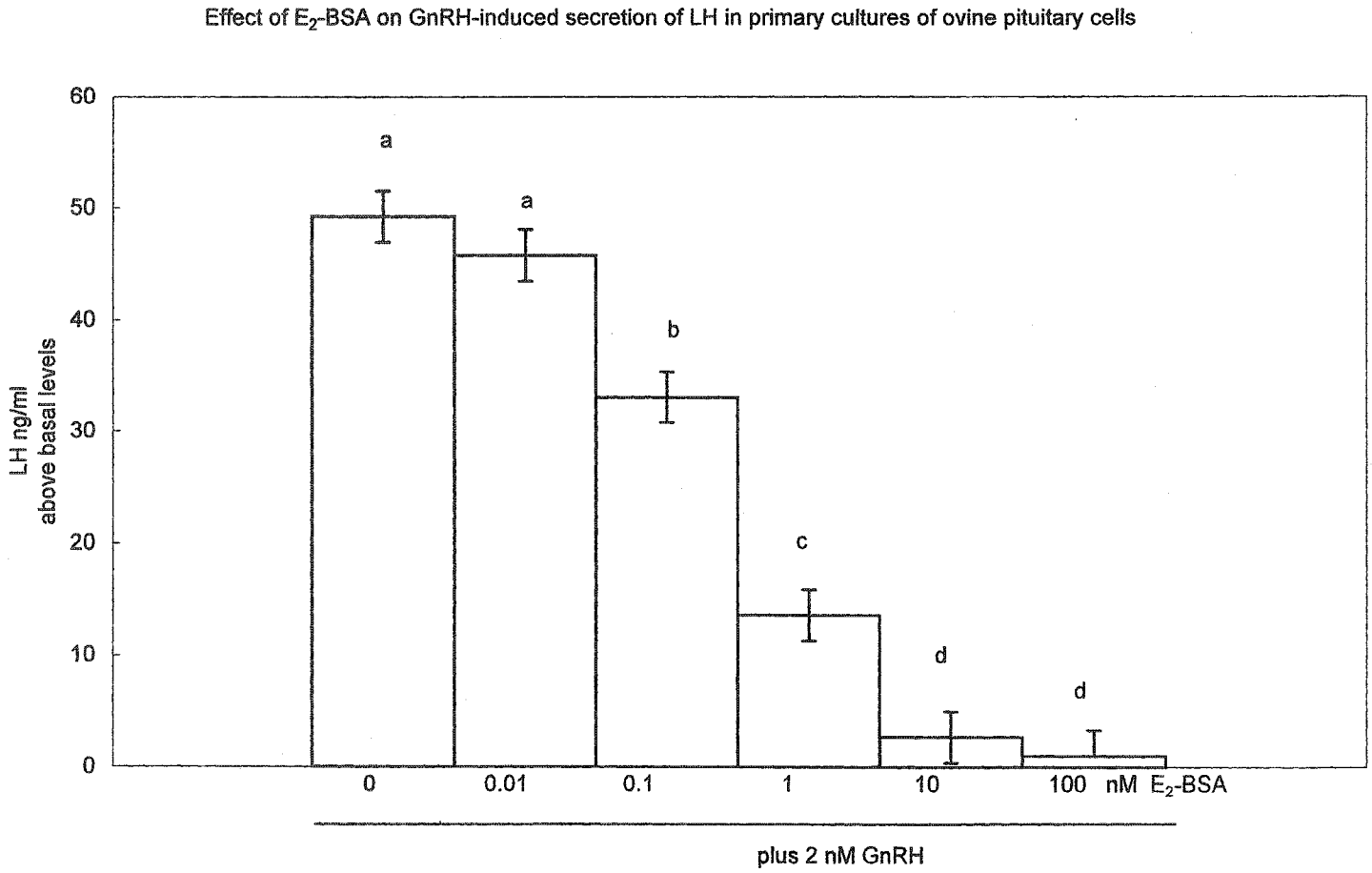


Figure 3.2. Cells (2 x 10^5 cells per well) were pre-incubated for 60 min with culture medium or E_2-BSA, washed and incubated for 15 min with the previous treatment plus 2 nM GnRH. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

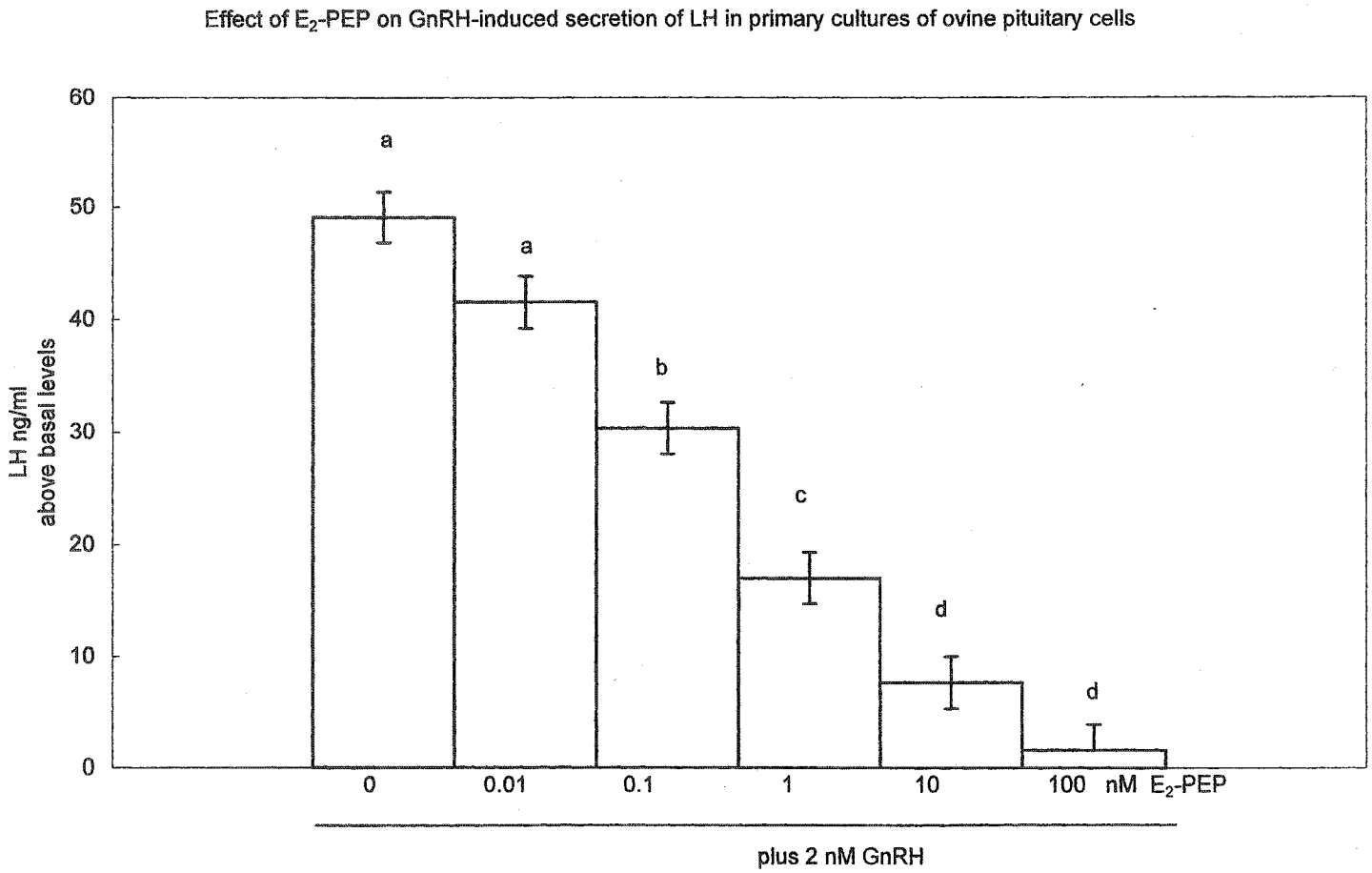


Figure 3.3. Cells (2 x 10^5 cells per well) were pre-incubated for 60 min with culture medium or E_2-PEP, washed and incubated for 15 min with the previous treatment plus 2 nM GnRH. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

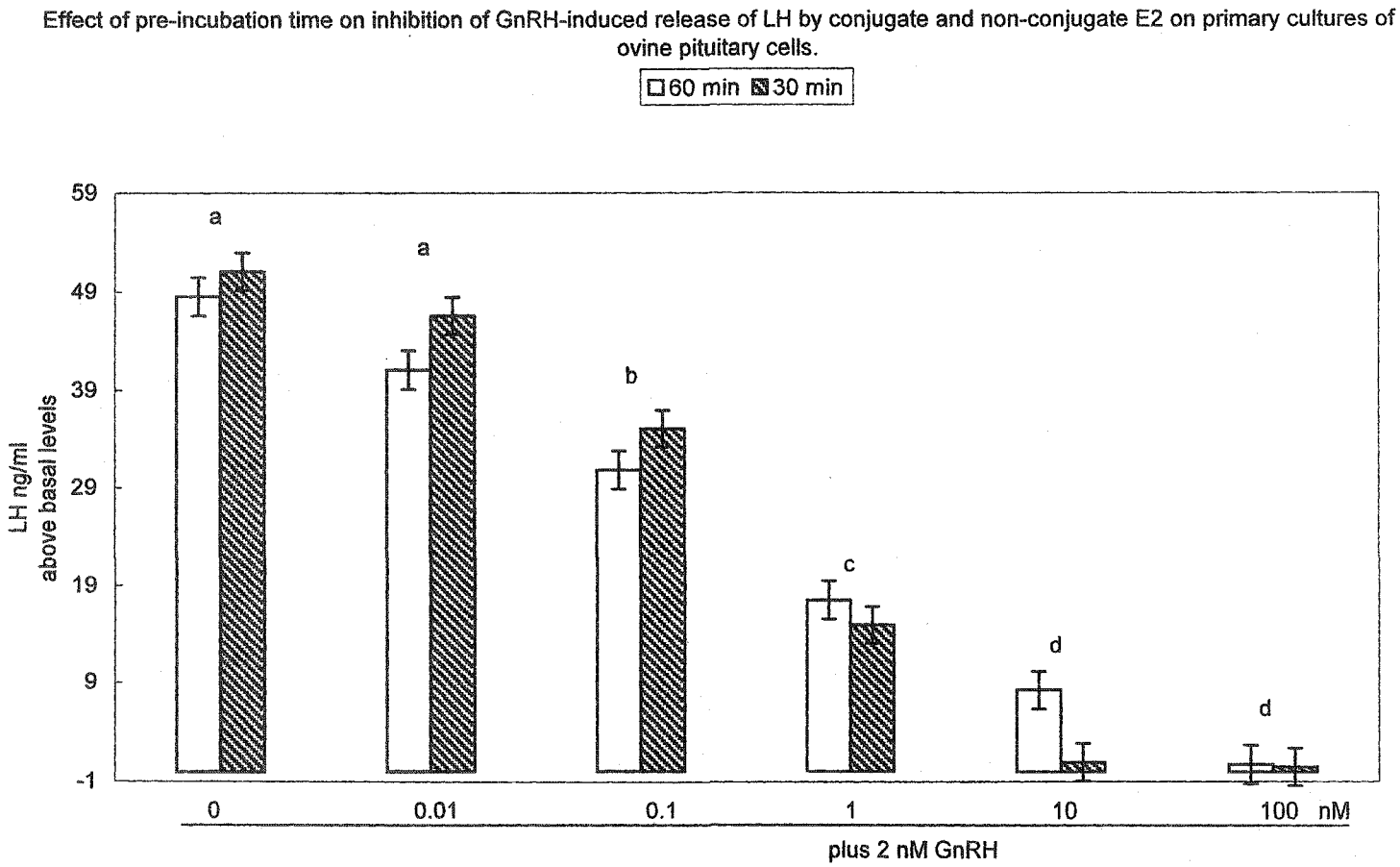


Figure 3.4. Cells (2 x 10^5 cells per well) were pre-incubated for 30 or 60 min with culture medium, E_2, E_2-BSA or E_2-PEP, washed and then incubated for 15 min with previous treatment plus 2 nM GnRH. Because no significant interactions between incubation time and dosage ($p > 0.1$) or between treatment and dosage ($p > 0.1$) were found, treatments were polled together. Comparisons were made within dosage. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

pre-incubation time was analyzed. Figure 3.4 shows that the dose-dependent inhibition of GnRH-induced secretion of LH by E_2, E_2-BSA, and E_2-PEP was not affected by pre-incubation time (30 versus 60 min). Similarly, pre-incubation time (15 versus 60 min) did not influence the inhibitory effect of E_2, E_2-BSA or E_2-PEP on GnRH-induced release of LH, and no significant interactions between pre-incubation time and dosage ($p > 0.1$) or between treatment and dosage ($p > 0.1$) were found; therefore, as mentioned above, treatments were pooled by dosage and the interaction of treatment with pre-incubation time was analyzed. Figure 3.5 shows that pre-incubation of pituitary cells for 15 or 60 min did not modify the magnitude of the dose-dependent inhibition of GnRH-induced release of LH induced by E_2, E_2-BSA or E_2-PEP.

Discussion. In previous studies from our laboratory, only about 25% of ovine pituitaries were responsive to GnRH. In the current study a slightly lower proportion of pituitaries (20%) were sensitive to GnRH, and there was a great variability in the magnitude of LH released after GnRH challenge from season to season. Since the objective of these experiments was to evaluate the effect of E_2 on GnRH-induced release of LH, only the GnRH-responsive pituitaries were suitable as experimental units. Several unsuccessful attempts were made to improve responsiveness to GnRH. Parameters tested in this effort included incubation in various culture media, different stocks of collagenase, dose of GnRH, and length of exposure to GnRH. Moreover, there was no association between physiological state of the ewe (intact versus OVX), or month within breeding season with sensitivity of pituitary cells to GnRH. Nevertheless, it is important to point out that in GnRH-sensitive pituitary cells E_2 consistently suppressed GnRH-induced release of LH.

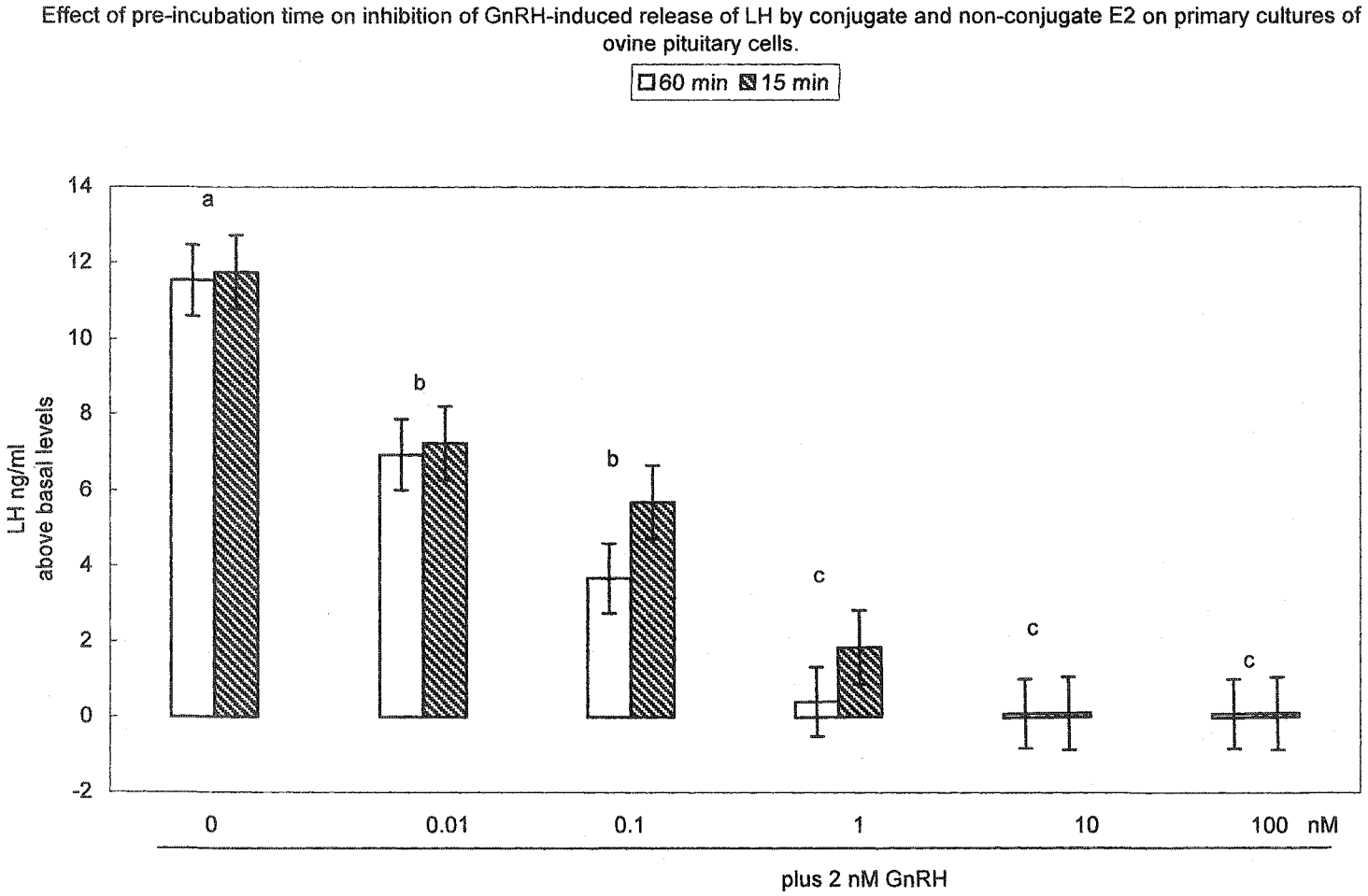


Figure 3.5. Cells (2×10^5 cells per well) were pre-incubated for 15 or 60 min with culture medium, E_2, E_2-BSA or E_2-PEP, washed and then incubated for 15 min with the previous treatment plus 2 nM GnRH. Because no significant interaction ocurred between incubation time and dosage ($p > 0.1$) or between treatment and dosage ($p > 0.1$), treatments were pooled. Comparisons were made within dosage. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

In the first experiment, pre-incubation of pituitary cells for 60 min with E_2 resulted in a dose-dependent inhibition of GnRH-induced release of LH; 0.1 nM E_2 resulted in a significant decreased ($p < 0.01$) in GnRH-induced release of LH but 10 nM E_2 was required to completely suppress release of LH. This is consistent with other reports where concentrations of E_2 in the range of 0.1 to 10 nM have been used successfully to modulate signaling pathways in hypothalamus (Prevot *et al.*, 1999), endothelial cells (Chambliss *et al.*, 2000), adipocytes (Santos *et al.*, 2002), neurons (Wong *et al.*, 2003), and a pituitary tumor cell line (Pappas *et al.*, 1994; Norfleet *et al.*, 2000). Similarly, 10 nM of either E_2-BSA or E_2-PEP completely prevented GnRH-induced release of LH. Likewise, 1 to 10 nM E_2-BSA has been used successfully to mimic acute actions of E_2 *in vitro* (Razandi *et al.*, 1999; Kim *et al.*, 1999; Razandi *et al.*, 2000a; Hisamoto *et al.*, 2001b; Wade *et al.*, 2001; Santos *et al.*, 2002; Wong *et al.*, 2003; Chen *et al.*, 2003).

Due to the limited number of dispersed cells obtained from ovine pituitaries, evaluation of the effect of pre-incubation time (15 and 30 min) on inhibition of LH secretion induced by conjugate or non-conjugate E_2 was carried out in separate experiments. A 60 min pre-incubation time was used in each experiment to serve as a basis for comparison. Pre-incubation of pituitary cells with E_2 or its conjugated forms for 15, 30 or 60 min induced a similar dose-dependent inhibition of GnRH-induced release of LH. However, the amount of LH released by GnRH in pituitaries used to compare 15 versus 60 min pre-incubation time was ~25% than the amount of LH released by GnRH in previous experiments (compare positive controls in Figures 3.4 and 3.5). In these less responsive pituitaries, basal levels of LH were 3-fold higher than the basal levels of LH

detected in normal sensitive cells. Although a lack or reduce responsiveness to physiological secretogogues, regarded to an enzyme-induced receptor damage, is commonly observed in enzymatically dissociated secretory cells (Kono, 1969; Amsterdam and Jamieson, 1974); it has been demonstrated that ovine pituitary cells fully recovered their ability to secrete LH in response to GnRH from 16 to 24 hours after enzymatic dispersion (Adams, 1979). In our study, cells were cultured for 48 h before challenge with GnRH, therefore a potential damage of GnRH receptors may not me the cause for the low sensitivity to GnRH. Whatever the reason for the low sensitivity to GnRH, it is reasonable to think that the high basal levels of LH may decrease the storage of LH in pituitary cells, resulting in a poor release of LH in response to GnRH.

As mentioned above, pre-incubation of pituitary cells with E_2 or its conjugated forms for 15, 30 or 60 min induced a similar dose-dependent inhibition of GnRH-induced release of LH. In several studies the treatment with E_2 or E_2-BSA over a wide range of intervals (1 to 40 min) modulated a variety of signaling pathways including cGMP production (Rusell *et al.*, 2000), activation of ERK (Santos *et al.*, 2002; Razandi *et al.*, 2000a), NOS (Kim *et al.*, 1999), and Akt (Hisamoto *et al.*, 2001b) among others. Therefore, our data support the concept that in ovine pituitary cells, E_2 acutely suppresses the GnRH-induced release of LH by a nongenomic mechanism. In this regard, several studies indicate that E_2-BSA, distinct from E_2, did not stimulate reporter activity in cells transfected with ERE-luciferase reporter constructs (Watters *et al.*, 1997; Razandi *et al.*, 1999; Razandi *et al.*, 2000b; Wade *et al.*, 2001) further suggesting that E_2-BSA acts through a nongenomic mechanism. Moreover, the novel synthesized E_2-PEP proved to be

biological active and mimic the acute suppression of GnRH-induced secretion of LH by E_2.

CHAPTER IV

CHARACTERIZATION OF THE ACUTE ACTION OF ESTRADIOL ON THE GnRH-INDUCED RELEASE OF LH IN PRIMARY CULTURES OF OVINE PITUITARY CELLS

Introduction. In the previous chapter, we demonstrated that E_2 and conjugated forms of E_2 acutely suppressed the GnRH-induced release of LH in a dose-dependent manner. Further characterization requires the determination of ERs in the mediation of acute actions of E_2. The use of ER modulators like tamoxifen or ICI 182,780 have proved that both genomic and nongenomic actions of E_2 are affected by these compounds, suggesting that both effects are mediated by similar if not the same ER. Additionally, the recent development of a new generation of selective steroid receptor modulators, which discriminate between the most common ER subtypes (ERα and ERβ) may provide new insight into the roles of ERα and ERβ in the mediation of nongenomic actions induced by E_2. We hypothesize that the acute action of E_2 on GnRH-induced release of LH are steroid and stereo specific, are mediated by ERs, and are differentially modulated by ERα and ERβ.

Materials and Methods

Preparation of Media and Stock Solutions. Dissociation and culture media were prepared as described in the previous chapter. Stock solutions of GnRH (in saline plus 0.1% BSA), E_2-BSA, and E_2-PEP (in saline solution) were stored at –20 °C in small aliquots. E_2, progesterone (P_4), testosterone (T), hydrocortisone (HC), 17α-estradiol

(αE_2), tamoxifen (Tx; Tocris, Inc.), 4-OH-tamoxifen (HTx; Tocris, Inc.), ICI 182,780 (ICI, Tocris, Inc.), E_2-Receptor (ER) α agonist (PPT; Tocris, Inc.), and ER β agonist (DPN; Tocris, Inc.), were freshly dissolved in ethanol the day of pituitary treatment.

Dissociation and Incubation of Pituitary Cells. Anterior pituitary tissue from OVX ewes was dispersed as described in the previous chapter. Cells were plated at 2×10^5 cells per well in 24-well tissue culture plates (Corning Glass Works), and incubated for 2 days at 37 °C under an atmosphere of 95% air: 5% CO_2. Cell viability was evaluated immediately after tissue dissociation and before administration of treatments by incubating the cells with 1% trypan blue for 3 to 4 minutes. Release of LH from primary cultures of ovine pituitary cells was quantified by radioimmunoassay (Niswender *et al.*, 1969).

Experimental Procedure. After 2 days incubation, anterior pituitary cells were washed twice with culture medium and treatments were applied in 1 ml of medium. Cells (2×10^5 cells per well; 4 wells per treatment, and 3 replicates (pituitaries)) were used for all treatments. To be certain that the inhibition of GnRH-induced release of LH induced by E_2 and conjugated forms of E_2 was not masked by the poor response to GnRH observed in most pituitaries in previous experiment, a set of cells were treated with serial dilutions of E_2, E_2-BSA or E_2-PEP as in the previous experiment was included as control. To evaluate the effect of other steroid hormones, and an E_2 stereoisomer (αE_2), cells were pre-incubated for 15 min with 0 or 1 nM E_2, or 100 nM P_4, T, HC or αE_2 with and without 1 nM E_2. After pre-incubation, cells were washed once with medium and incubated for 15 min with the previous treatment plus 2 nM GnRH. To study the effect of E_2 antagonists, cells were pre-incubated for 15 min with 0 or 1 nM E_2 or 100 nM Tx,

HTx or ICI with and without 1 nM E_2, E_2-BSA or E_2-PEP. After pre-incubation, cells were washed once with medium and incubated for 15 min with culture medium (negative control), or the previous treatment plus 2 nM GnRH. To evaluate the effect of specific ER (α and β) agonists, cells were pre-incubated for 15 min with 0, 0.01, 0.1, 1, 10 or 100 nM PPT or DPN. After pre-incubation, cells were washed once with medium and incubated for 15 min with culture medium (negative control), or the previous treatment plus 2 nM GnRH.

Statistical Analysis. Data were subject to analysis of variance (ANOVA) using the general linear model of SAS (1987) in a completely randomized design. When a significant F-value was obtained means were separated using LSD adjusted by Tukey's procedure.

Results. The dissociation procedure yielded 79.9 ± 0.45 x 10^6 cells per pituitary (n=3). Viability of cultured pituitary cells after tissue dissociation was always greater than 94% (95.1 ± 0.8%; n=3). After incubation for 2 day, cell viability was always greater than 80% (87.1 ± 1.3; n=3). Coefficient of variation, intra- and inter-assay (n=6) for LH were 4% and 9%, respectively; and the minimum detectable amount of LH averaged 24 pg. Incubation of anterior pituitary cells with 2 nM GnRH for 15 min increased release of LH compared with untreated cells (33.5 ± 2.6 versus 18.76 ± 2.6 ng/ml; $p < 0.01$). Application of E_2 (Figure 4.1), E_2-BSA (Figure 4.2) or E_2-PEP (Figure 4.3) to cultured cells caused an inhibition of GnRH-induced release of LH ($p < 0.01$). For E_2 treated cells, 1 nM E_2 decreased release of LH compared with GnRH treated cells to levels found in untreated cells (Figure 4.1). Similarly, 10 nM E_2-BSA (Figure 4.2) or E_2-

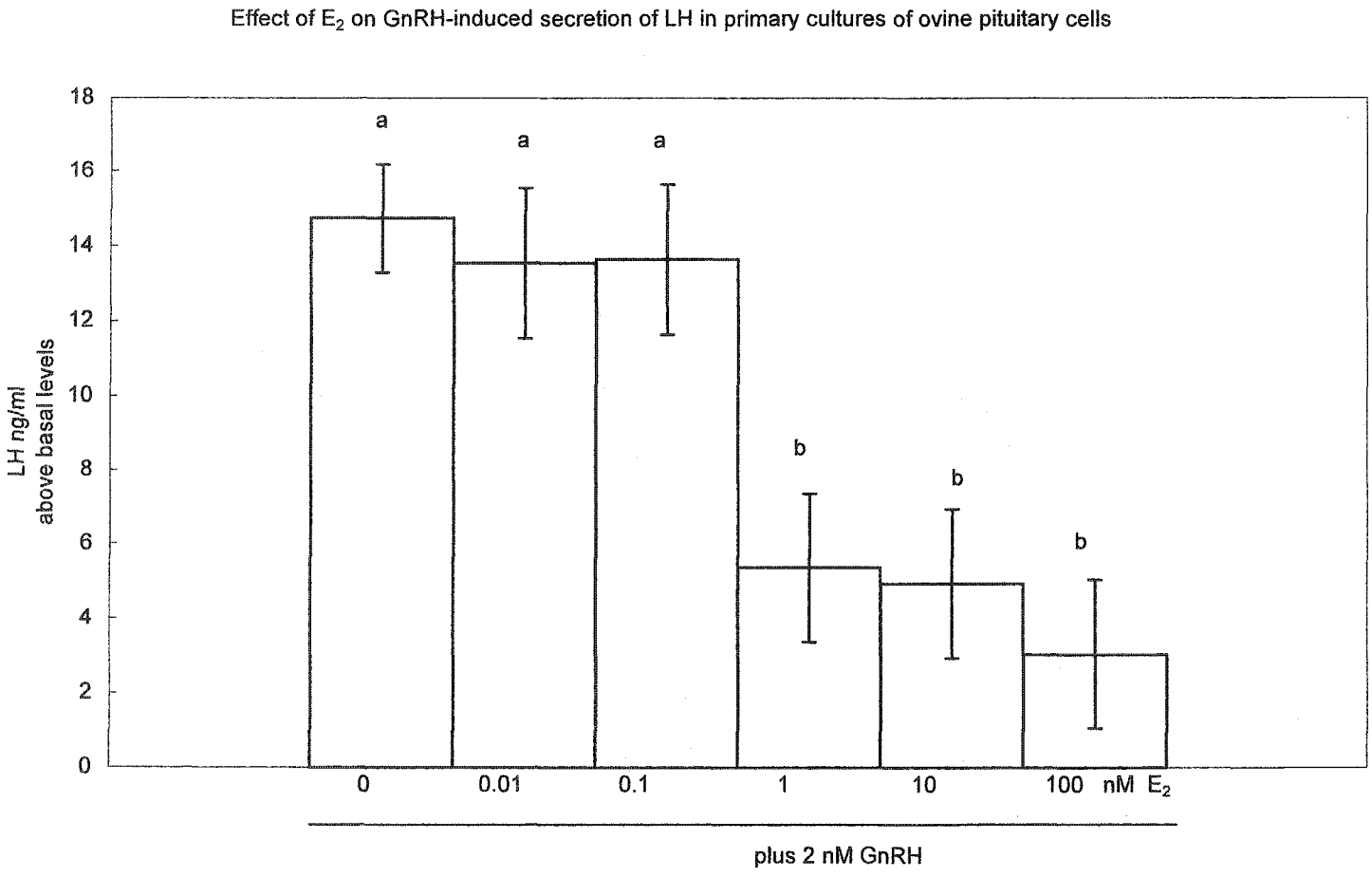


Figure 4.1. Cells (2×10^5 cells per well) were pre-incubated for 15 min with culture medium or E_2, washed and incubated for 15 min with the previous treatment plus 2 nM GnRH. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

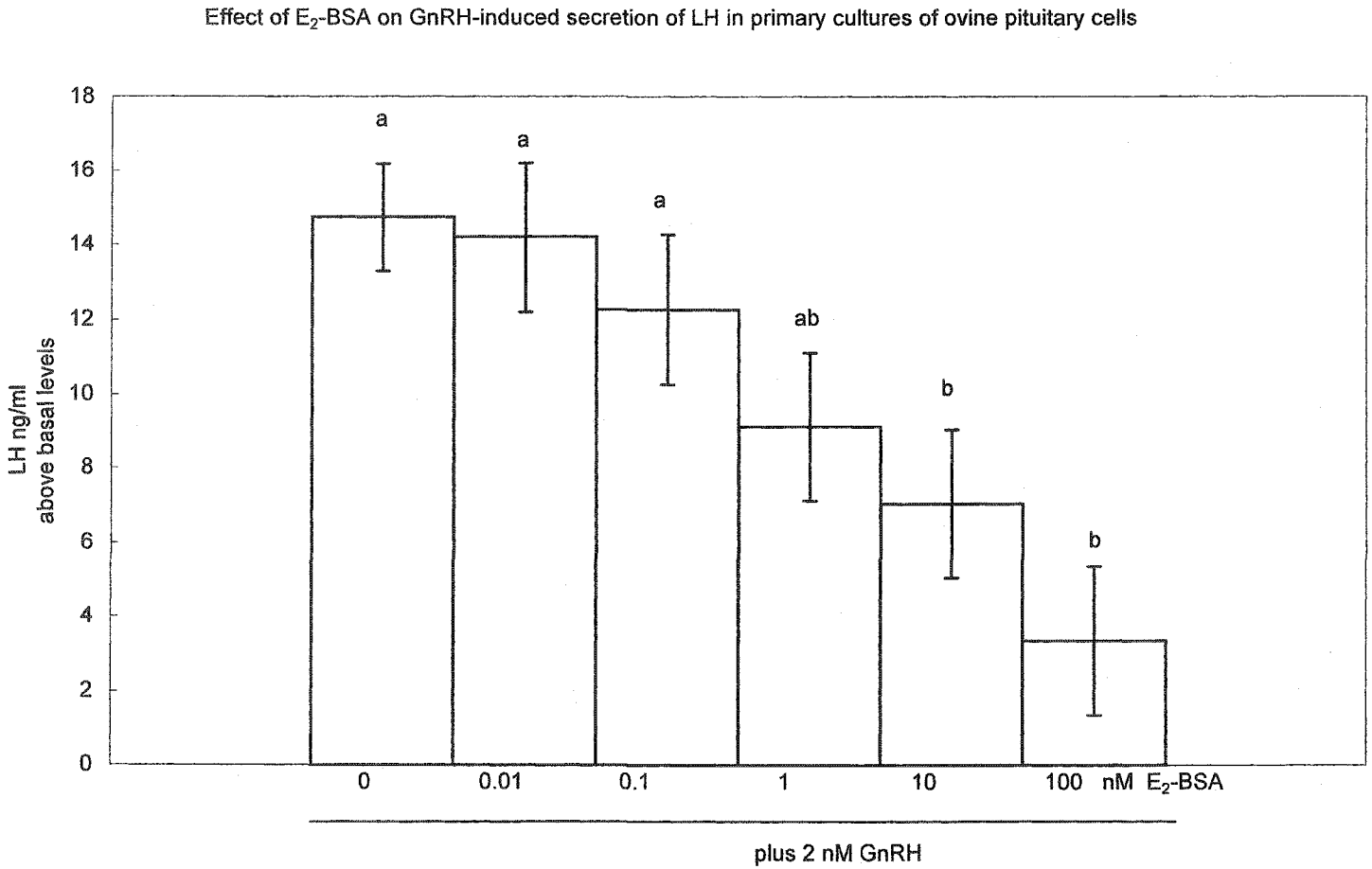


Figure 4.2. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium or E_2-BSA, washed and incubated for 15 min with the previous treatment plus 2 nM GnRH. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

PEP (Figure 4.3) decreased LH release to levels found in untreated cells. Treatment of pituitary cells with steroid hormones (100 nM P_4, T, HC or αE_2) did not affect the GnRH-induced release of LH (Figure 4.4). Moreover, when pituitary cells were co-incubated with these steroids plus E_2, they did not alter the inhibition of GnRH-induced release of LH caused by 1 nM of E_2 (Figure 4.5). Similarly, treatment of pituitary cells with E_2 antagonists did not influence the GnRH-induced release of LH (Figure 4.6). However, E_2 antagonists prevented ($p < 0.01$) the inhibition of GnRH-induced release of LH induced by 1 nM E_2 (Figure 4.7). In this experiment, and different from those performed earlier, 1nM of E_2-BSA (Figure 4.8) or E_2-PEP (Figure 4.9) did not cause a clear inhibition of GnRH-induced release of LH. Because the corresponding treatments within E_2-BSA and E_2-PEP treatments were not different, data from both conjugates were analyzed together. In this case, E_2 conjugates decreased ($p < 0.02$) GnRH-induced release of LH, but like with E_2, the presence of E_2 antagonists prevented the inhibition of GnRH-induced release of LH (Figure 4.10).

The selective ERα agonist, PPT, decreased ($p < 0.01$) release of LH in response to a GnRH challenge but only at a concentration of 100 nM (Figure 4.11), whereas the selective ERβ agonist, DPN, did not decrease ($p > 0.1$) GnRH-induced release of LH at the concentrations tested (Figure 4.12).

Discussion. As in the last set of experiments discussed in previous chapter, the sensitivity of pituitary cells to a GnRH challenge was lower and accompanied by higher basal levels of LH compared with the more responsive cells initially used. Treatment of pituitary cells with E_2, E_2-BSA or E_2-PEP did not induced the smooth dose-dependent inhibition of GnRH-induced release of LH observed in more sensitive cells, instead a

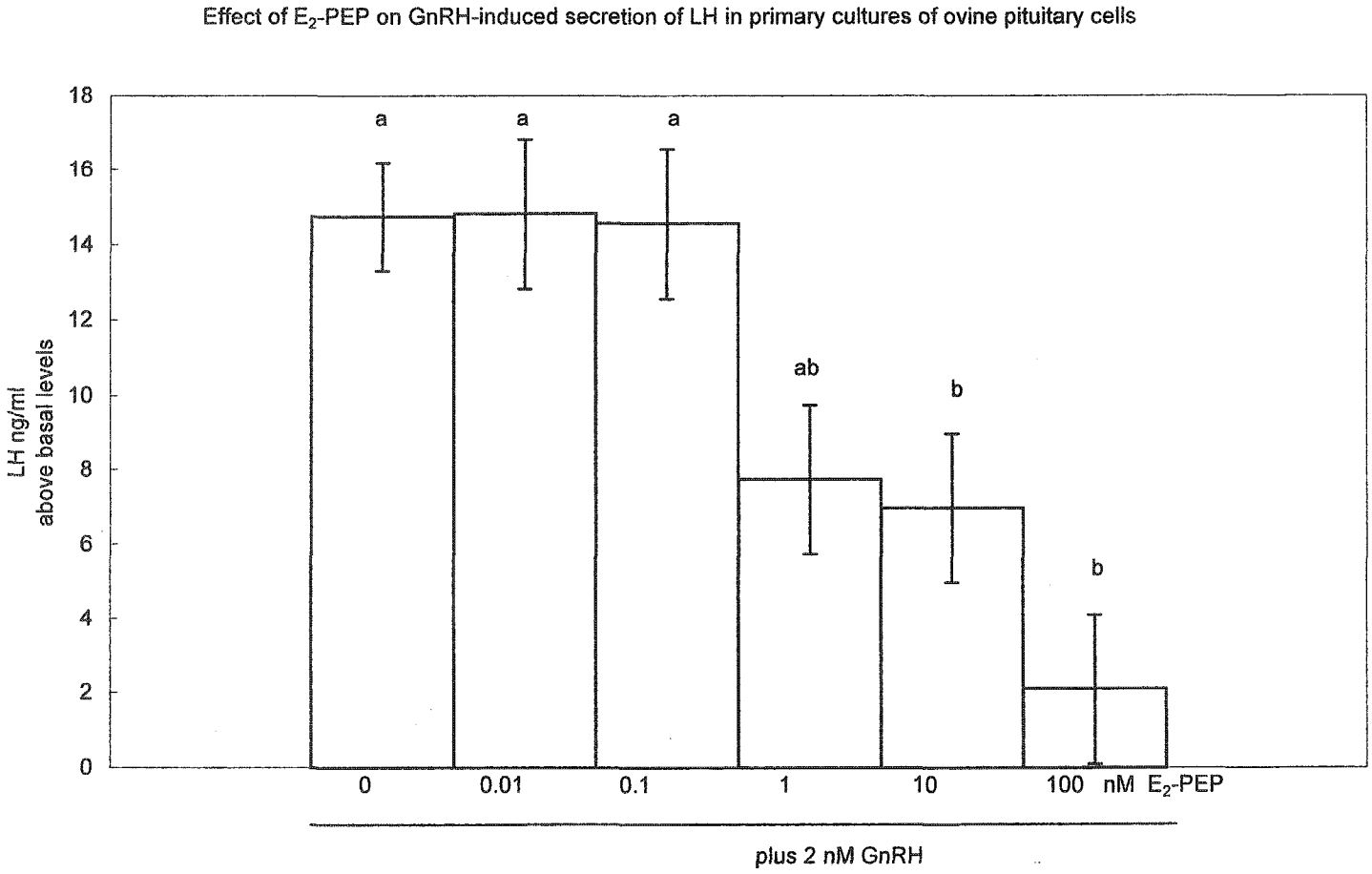


Figure 4.3. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium or E_2-PEP, washed and incubated for 15 min with the previous treatment plus 2 nM GnRH. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; $p < 0.01$; mean ± standard error.

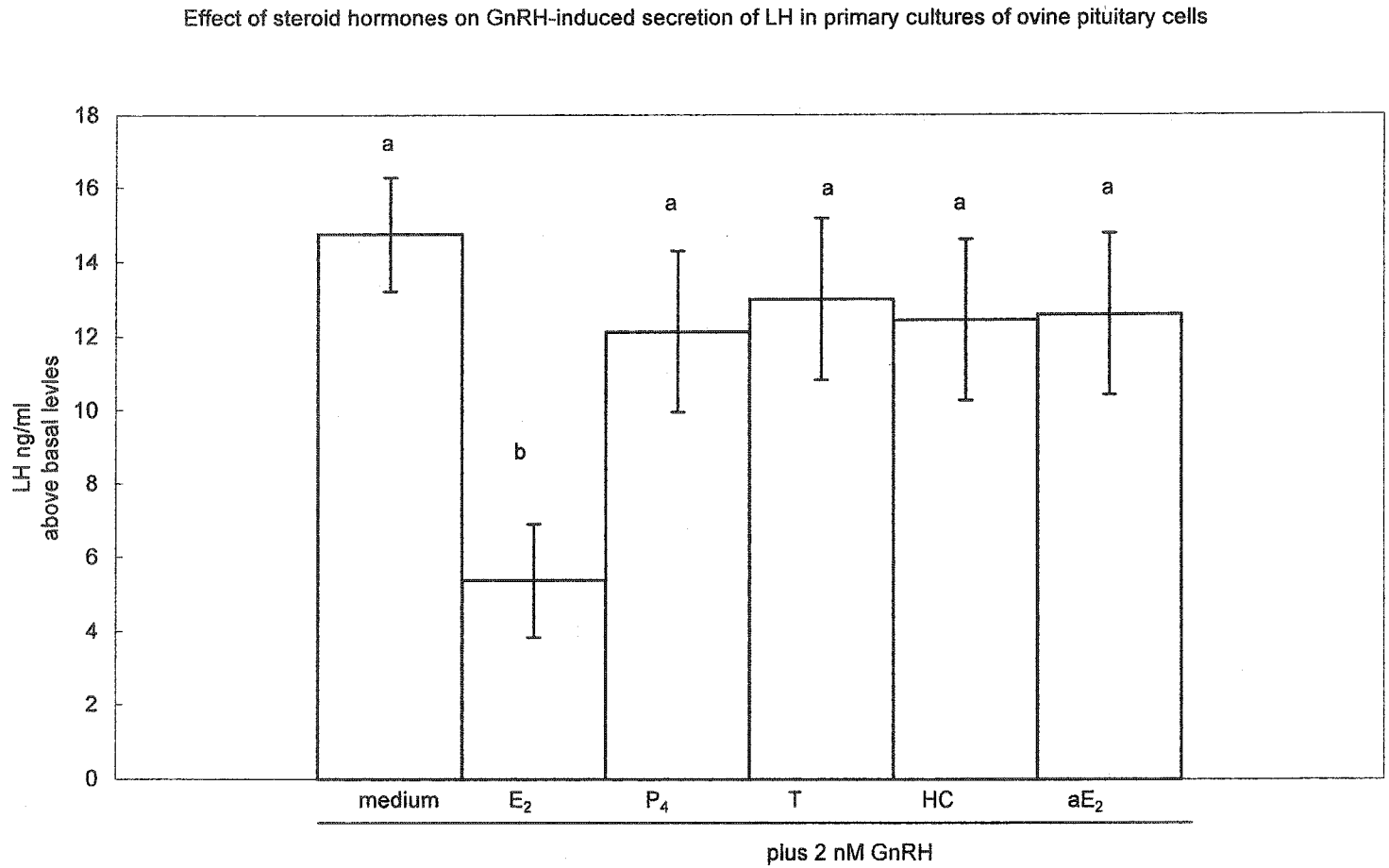


Figure 4.4. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM 17 β estradiol (E_2) or 100 nM of one of the following steroids; progesterone (P_4), testosterone (T), hydrocortisone (HC), or 17 α estradiol (αE_2) . Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 2 nM GnRH or culture medium. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.01$

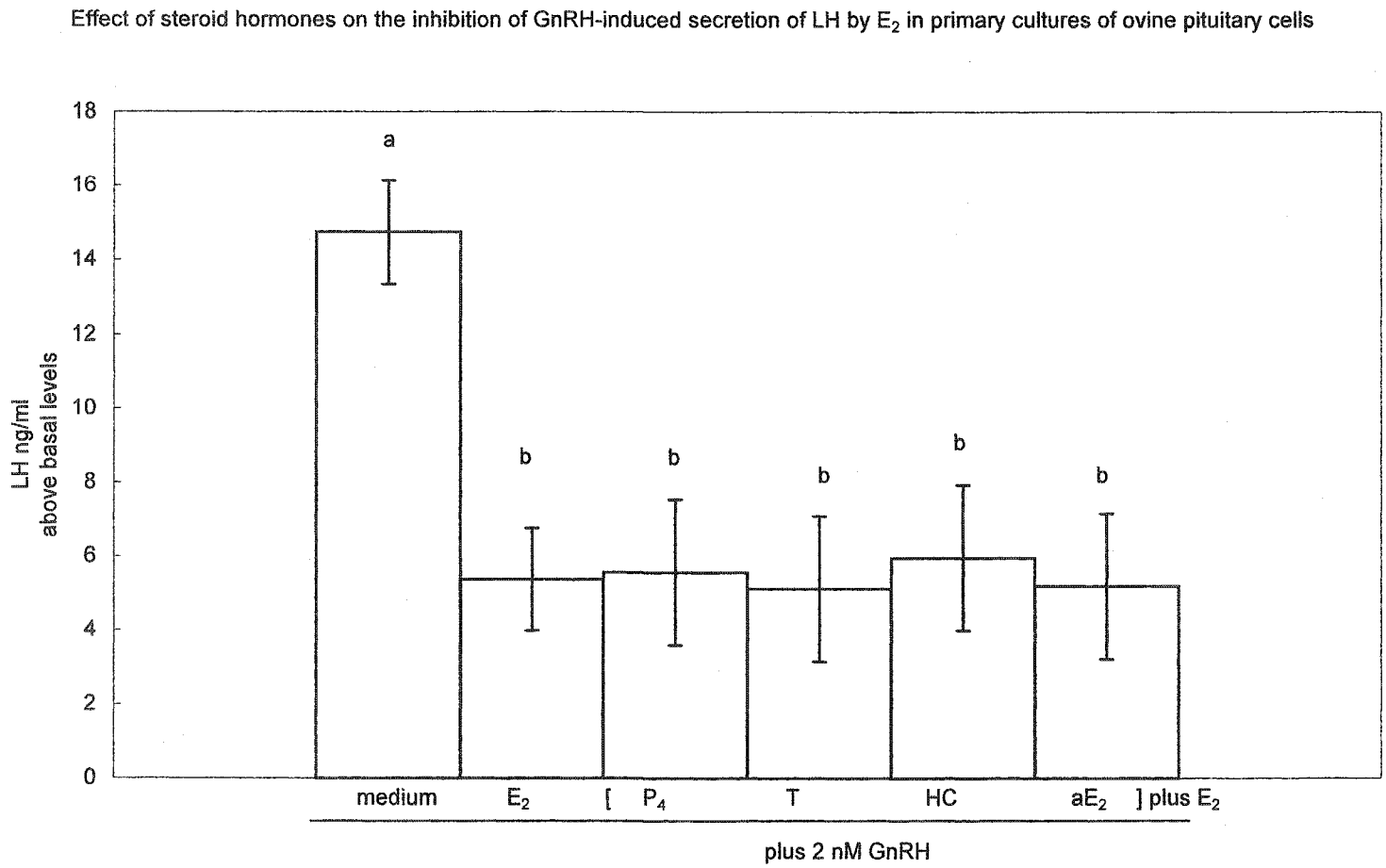


Figure 4.5. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM 17 β estradiol (E_2) or 100 nM of one of the following steroids; progesterone (P_4), testosterone (T), hydrocortisone (HC), or 17 α estradiol (αE_2). Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 1 nM E_2 and 2 nM GnRH or culture medium in the control groups. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.01$

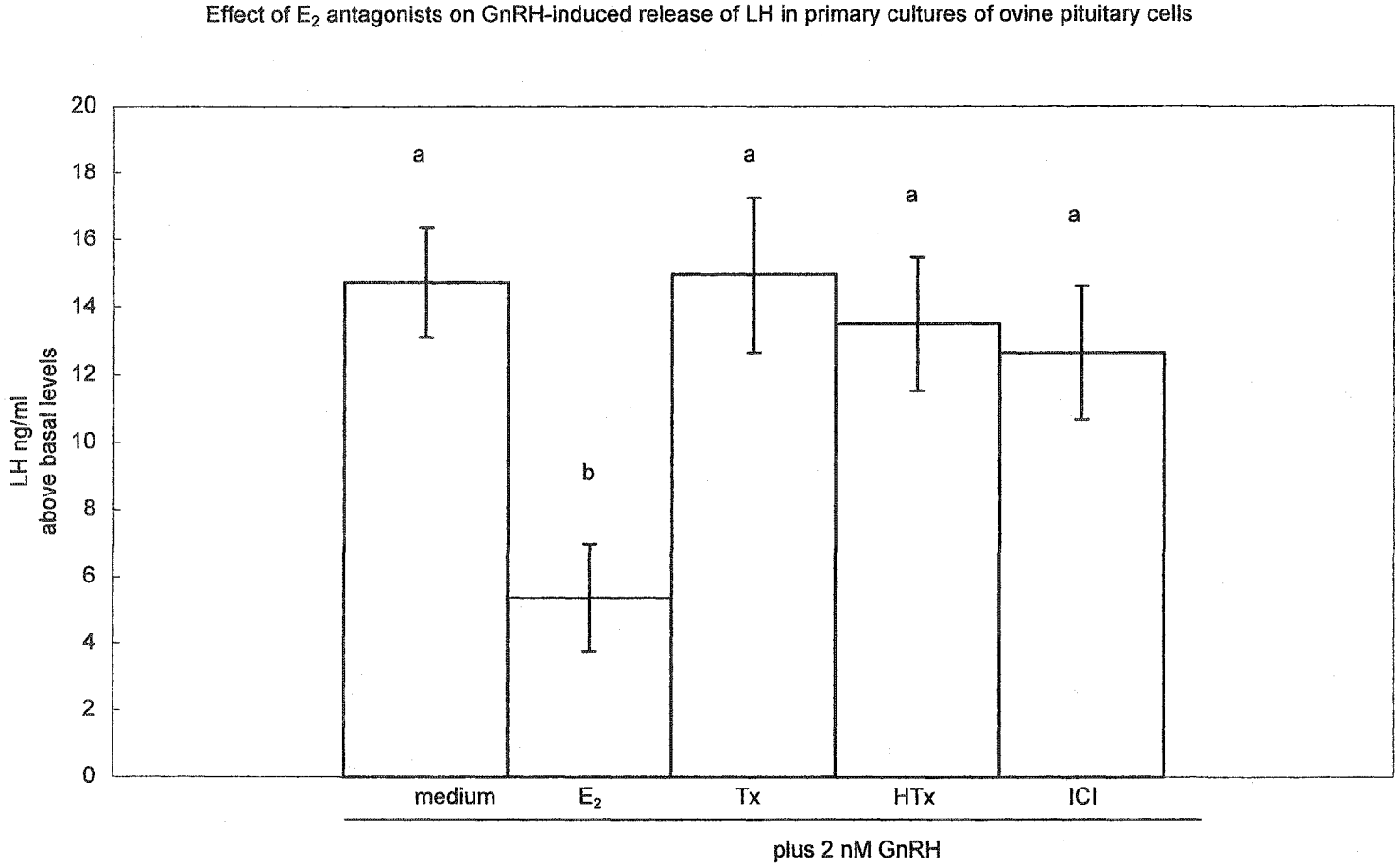


Figure 4.6. Cells (2×10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM 17 β estradiol (E_2) or 100 nM of one of the following E_2 antagonists; tamoxifen (Tx), hydroxytamoxifen (HTx), or ICI 182,780 (ICI) . Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.01$

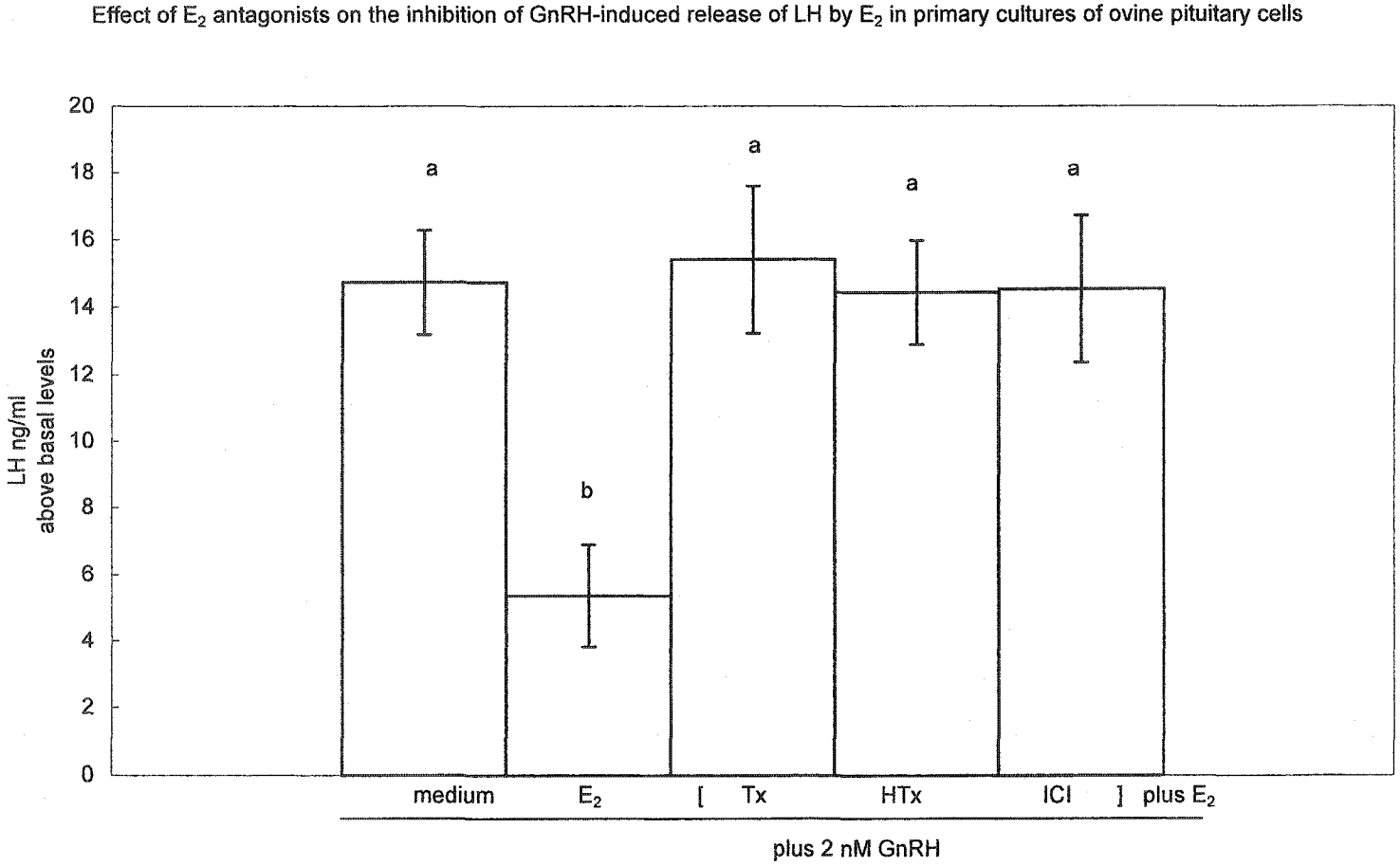


Figure 4.7. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM 17 β estradiol (E_2) or 100 nM of one of the following E_2 antagonists; tamoxifen (Tx), hydroxytamoxifen (HTx), or ICI 182,780 (ICI). Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 1 nM E_2 and 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.01$

Effect of E_2 antagonists on the inhibition of GnRH-induced release of LH by E_2-BSA in primary cultures of ovine pituitary cells

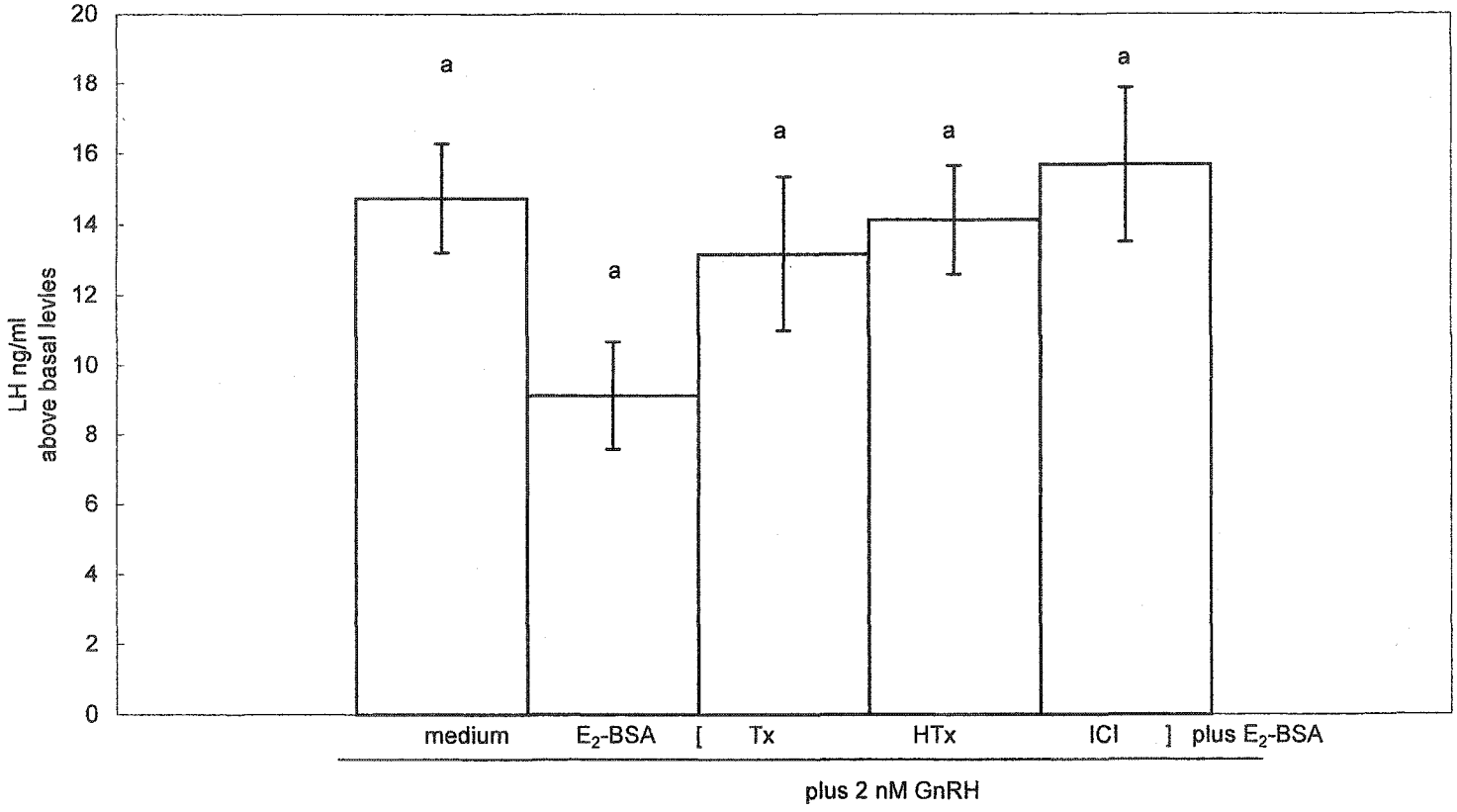


Figure 4.8. Cells (2×10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM E_2-BSA or 100 nM of one of the following E_2 antagonists; tamoxifen (Tx), hydroxytamoxifen (HTx), or ICI 182,780 (ICI). Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 1 nM E_2-BSA and 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.01$

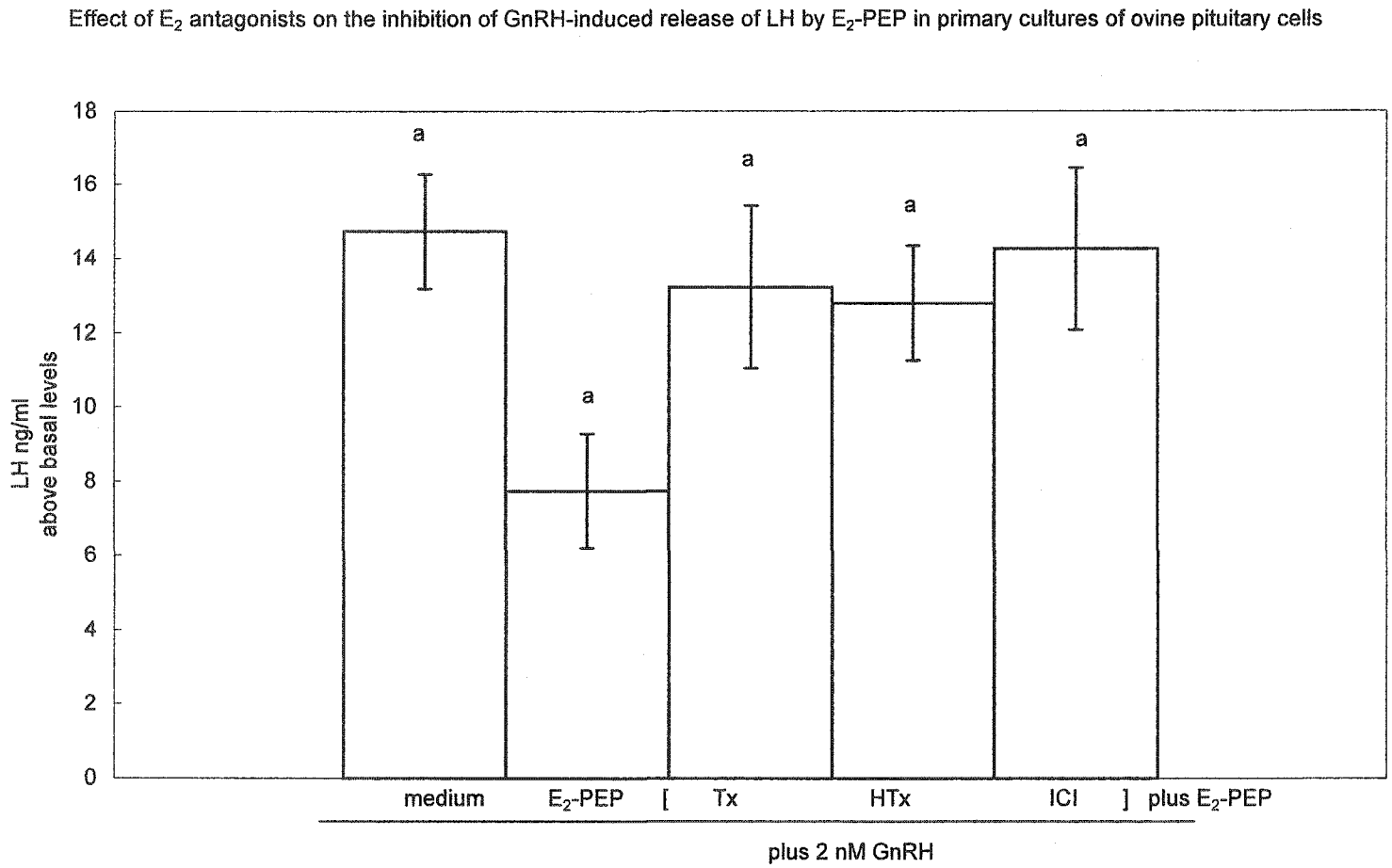


Figure 4.9. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM E_2-PEP or 100 nM of one of the following E_2 antagonists; tamoxifen (Tx), hydroxytamoxifen (HTx), or ICI 182,780 (ICI). Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 1 nM E_2-PEP and 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments;mean ± standard error; $p < 0.01$

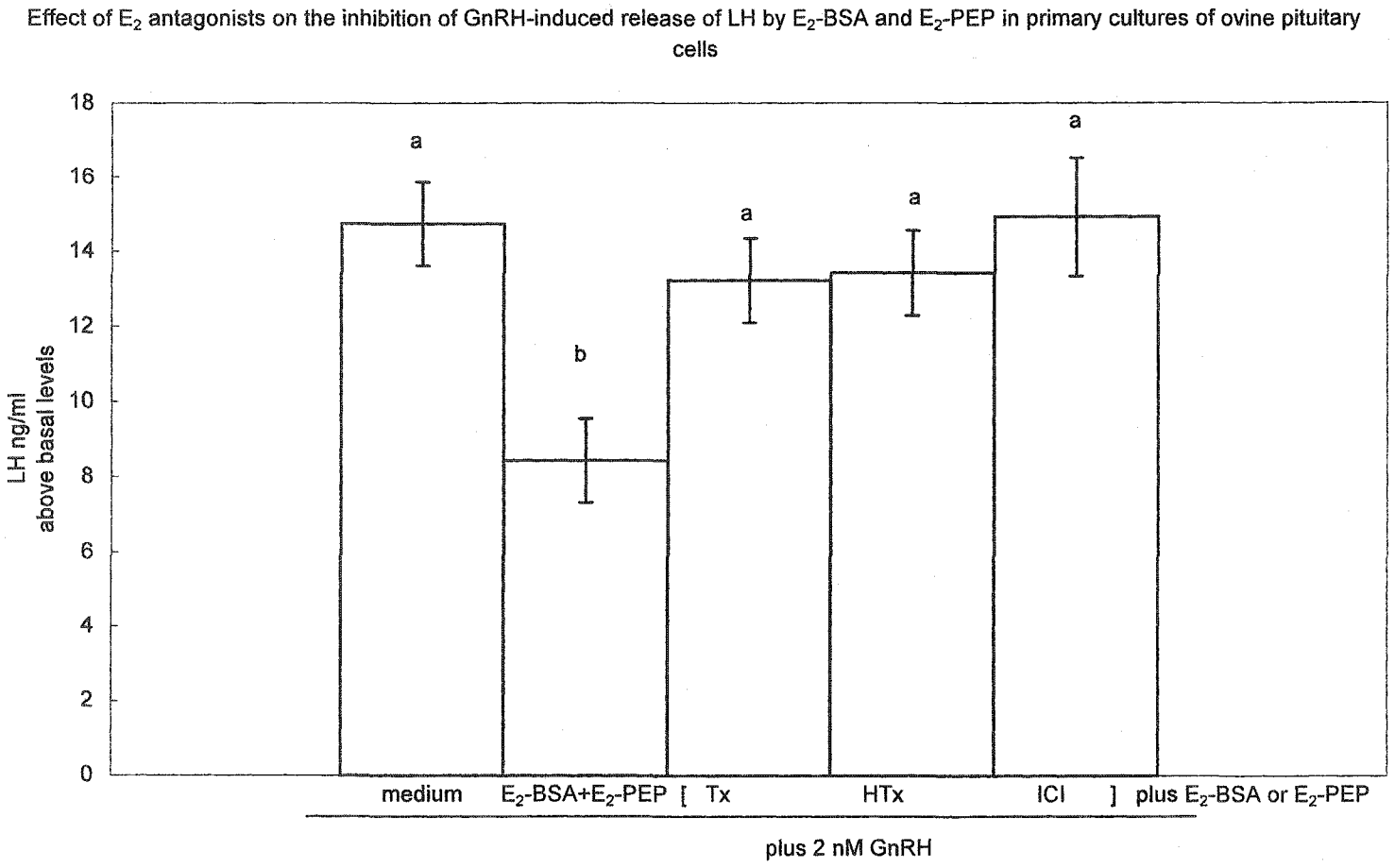


Figure 4.10. This figure shows polled data from figures 16 and 17. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with culture medium, 1 nM of either conjugated form of E_2 or 100 nM of one of the following E_2 antagonists; tamoxifen (Tx), hydroxytamoxifen (HTx), or ICI 182,780 (ICI). Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 1 nM E_2-PEP and 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.02$

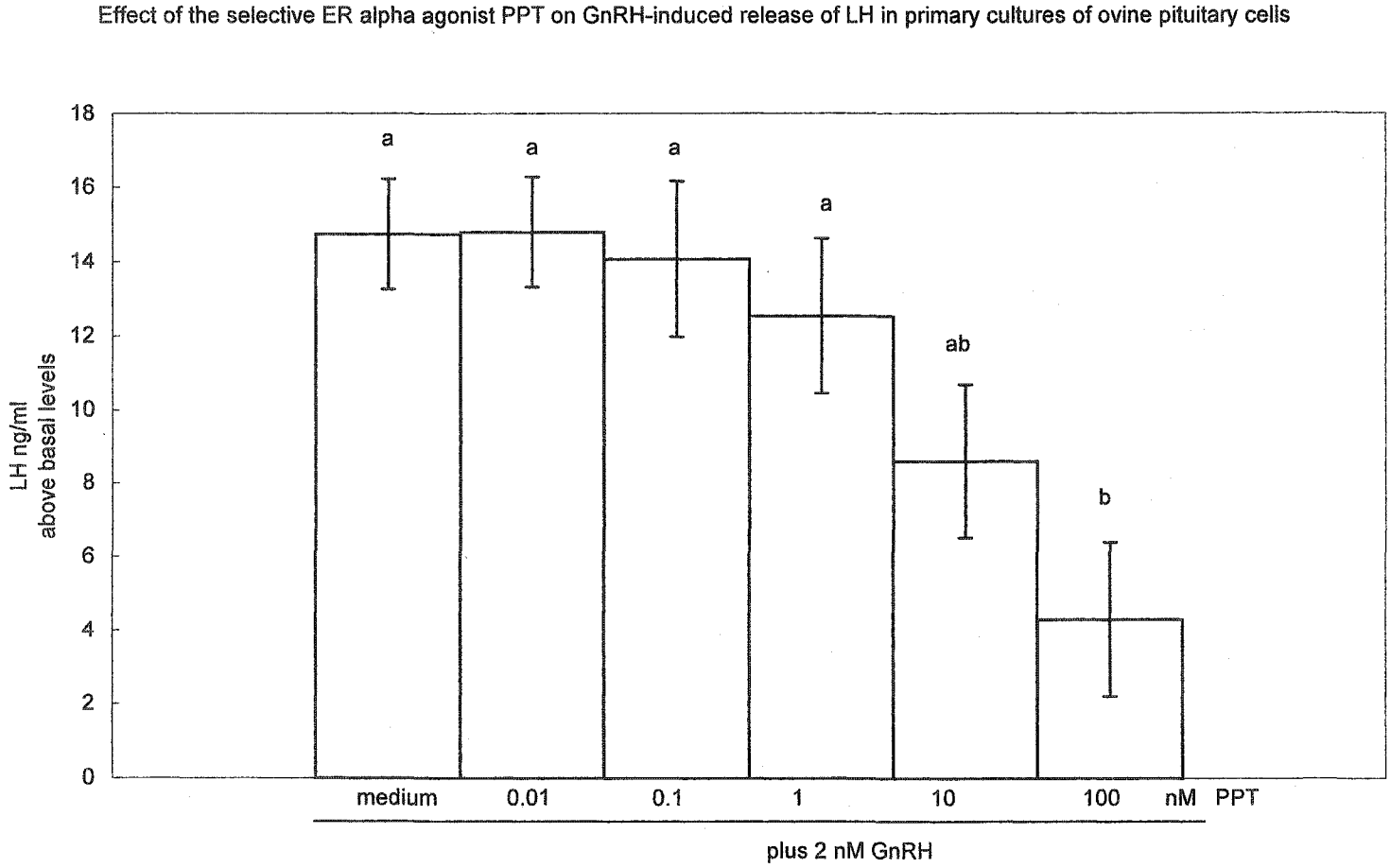


Figure 4.11. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with the ER alpha selective agonist PPT from 0 to 100 nM. Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparisons controls v.s. treatments; mean ± standard error; $p < 0.01$

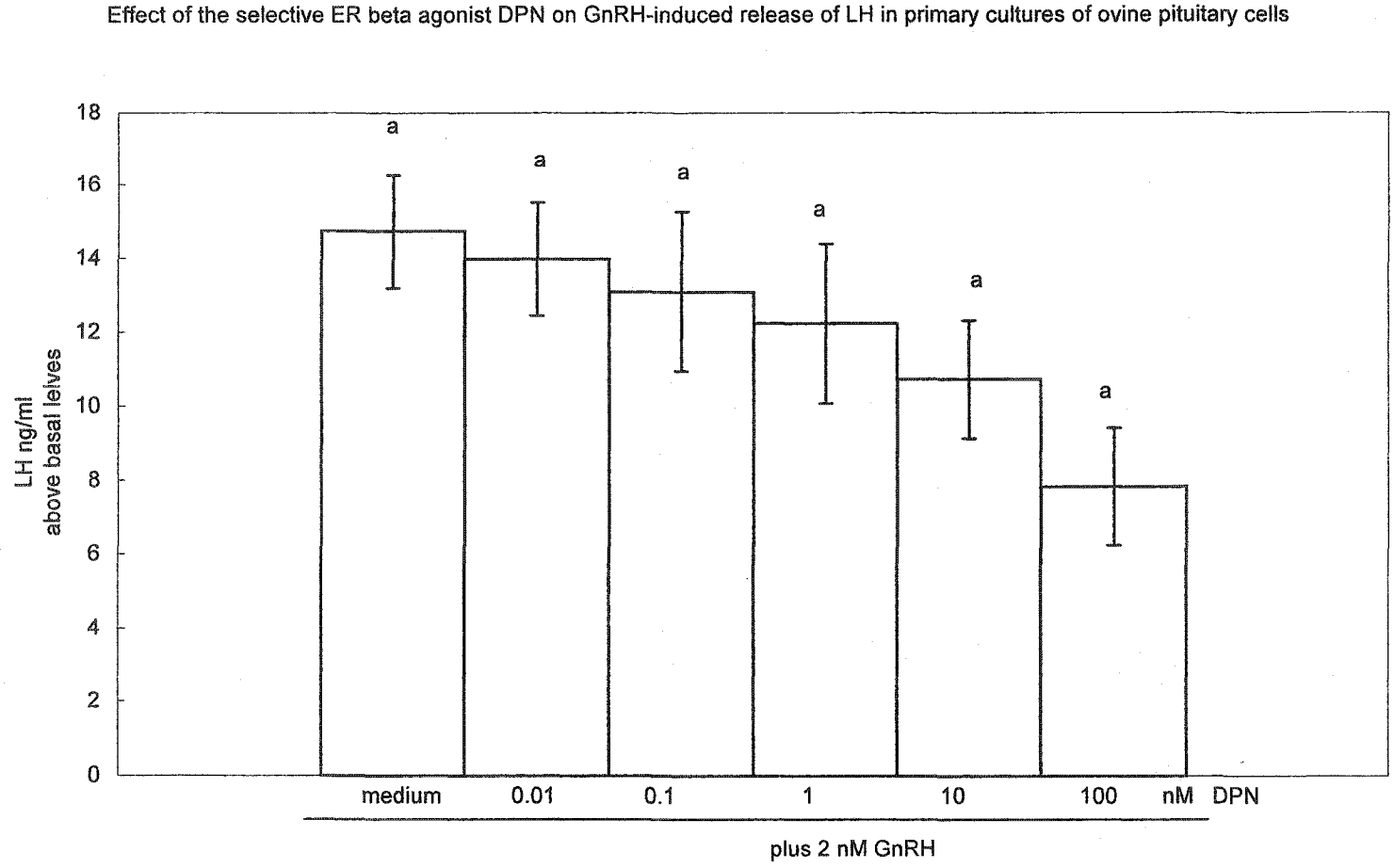


Figure 4.12. Cells (2 x 10^5 cells per well) were pre-incubated for 15 min with the ER alpha selective agonist DPN from 0 to 100 nM. Culture medium was aspirated and cells incubated for 15 minutes with culture medium containing the previous treatment plus 2 nM GnRH or culture medium in the control group. Data represent the average of 3 pituitaries; 3 or 4 wells per treatment; comparations controls v.s. treatments; mean ± standard error; $p > 0.1$

sudden decrease in LH release to the media was detected at 1 or 10 nM of unconjugated or conjugated E_2, respectively.

As expected, incubation of pituitary cells with 100-fold excess of progesterone, testosterone, hydrocortisone or the stereoisomer 17α-estradiol did not mimic the acute inhibition of E_2 on GnRH-induced release of LH. This suggests that inhibition of GnRH-induced release of LH is a specific action of E_2 and agrees with previous reports where other steroid hormones failed to mimic the acute actions of E_2 on activation of NOS, (Goetz *et al.*, 1999; Prevot et al., 1999) and several protein kinases (Singer *et al.*, 1996; Watters *et al.*, 2000; Razandi *et al.*, 2000a; Razandi *et al.*, 2000b; Santos *et al.*, 2002; Wong *et al.*, 2003), as well as cAMP production (Aronica *et al.*, 1994). Furthermore, in our study, addition of 100-fold excess of the steroid hormone tested did not prevent inhibition by E_2 of the GnRH-induced release of LH, further supporting the specificity of this effect. Similarly, in most studies17α-estradiol did not activate signaling pathways acutely stimulated by E_2 (Chambliss *et al.*, 2000; Watters *et al.*, 2000; Razandi *et al.*, 2000a; Hisamoto *et al.*, 2001b; Santos *et al.*, 2002).

The ability of ER synthetic ligands to prevent the inhibition of GnRH-induced secretion of LH by E_2 proved that 100-fold excess of anti-estrogenic compounds completely prevented the binding of E_2 to ERs, further support the hypothesis that the acute action of E_2 occurs via ERs, which agrees with numerous others reports where tamoxifen, 4-OH-tamoxifen, and ICI 182,780 prevented the acute action of E_2 on modulation of signaling pathways including activation of NOS (Chen Z *et al.*, 1999; Goetz *et al.*, 1999; Kim *et al.*, 1999 Simoncini and Genazzani, 2000;Russell *et al.*, 2000; Hisamoto *et al.*, 2001a; Hisamoto *et al.*, 2001b; Chen *et al.*, 2003), MAP kinase cascades

(Singer *et al.*, 1996; Migliaccio *et al.*, 1996; Razandi *et al.*, 1999; Singer *et al.*, 1999; Razandi *et al.*, 2000a; Watters *et al.*, 2000; Wade *et al.*, 2001; Razandi *et al.*, 2002; Song *et al.*, 2002; Pedram *et al.*, 2002; Santos *et al.*, 2002; Mize *et al.*, 2003), and tyrosine kinases (Migliaccio *et al.*, 1996; Song *et al.*, 2002), PI3K (Stoica *et al.*, 2002), as well as phosphorylation of transcription factors (Razandi *et al.*, 2000a; Santos *et al.*, 2002; Song *et al.*, 2002; Wade *et al.*, 2003). The fact that anti-estrogenic compounds did not cause an acute effect on cellular response when administered alone, agrees with previous reports (Bourassa *et al.*, 1996; Chen Z *et al.*, 1999; Goetz *et al.*, 1999) and further support the idea that the binding of ERs to these compounds prevents their occupancy by E_2, resulting in a rapid antagonistic action. Likewise, in our study, anti-estrogenic compounds prevented the inhibition of GnRH-induced release of LH by conjugated forms of E_2. An antagonistic action of tamoxifen and ICI 182,780 on the mimetic action of E_2-BSA in signaling pathways rapidly activated by E_2 have been reported. For example, the rapid release of NO by E_2-BSA from median eminence fragments was prevented by tamoxifen (Prevot *et al.*, 1999); whereas the E_2-BSA-induced phosphorylation of MAP kinases was prevented by ICI 182,780 (Razandi *et al.*, 2000a; Wade *et al.*, 2001; Santos *et al.*, 2002).

It has been proposed that in some cells the acute actions of E_2 in cellular function may be mediate by a different binding protein than the common ER α and β (Gu *et al.*, 1999; Singh *et al.*, 2000; Toran-Allerand *et al.*, 2002). In this scenario ICI 182, 780 appears to behave atypically and it has been interpreted as a suggestive of binding to a novel binding protein (Gu *et al.*, 1999; Singh *et al.*, 2000; Toran-Allerand *et al.*, 2002; Wong *et al.*, 2003). For example, in immature neural cells, ICI 182,780 mimicked the

acute actions of E_2 on cAMP/PKA activation (Watters and Dorsa, 1998) and phosphorylation of ERK 1/2 (Wong *et al.*, 2003); whereas in neural cells from ERKO mice, ICI 182,780 did not antagonize the acute actions of E_2 on ERK phosphorylation (Singh *et al.*, 2000) nor the E_2-induced potentiation of kainate currents (Gu *et al.*, 1999). Interestingly, different than the observed in our study, when ICI 182,780 behaved atypically, 17α-E_2, also mimicked actions of E_2 (Singh *et al.*, 2000; Wong *et al.*, 2003).

Although ICI 182,780 is not considered a selective ER antagonist, the fact that in neural cells from ERKO mice ICI 182,780 did not prevent, for example, the E_2-induced phosphorylation of ERK (Singh *et al.*, 2000) suggests that at least some of the antagonistic actions of ICI 182,780 occur via ERα. The role of ER subtypes in the mediation of the inhibition of GnRH-induced release of LH by E_2 was further evaluated by using selective agonists for ERα or ERβ. Our data agrees with previous observation, implicating ERα in the mediation of the acute inhibition induced by E_2. The selective agonists PPT and DPN have been shown to selectively recruit co-activators via ERα or ERβ, respectively (Kraichely *et al.*, 2000); however, their ability to mimic acute actions of E_2 has just begun to be investigated. In a recent study, PPT acutely mimicked the vasodilatory action of E_2 in mesenteric arteries; however, when DPN was given at pharmacological concentrations it also had and smaller but significant effect on vasodilation (Montgomery *et al.*, 2003).

In conclusion, the acute inhibition of GnRH-induced release of LH observed in pituitary cells after treatment with E_2 proved to be steroid specific. The actions of E_2, E_2-BSA or E_2-PEP in secretion of LH were equally prevented by antagonistic compounds of ER, which suggest that ERs mediate this action. Moreover, the use of selective ER

agonists indicates that acute inhibition of GnRH-induced release of LH by E_2 occurs via ERα.

CHAPTER V

EFFECT OF ESTRADIOL AND TWO CONJUGATED FORMS OF ESTRADIOL ON GONADOTROPIN SECRETION IN OVARIECTOMIZED EWES

Introduction. Once the acute action of E_2 and its conjugated forms was characterized *in vitro*, the next step was to characterize their effects on gonadotropin secretion in an *in vivo* model. As discussed in the introductory chapter, the effect of E_2 on secretion of LH is characterized by a rapid decrease in secretion of LH, followed several hours later by a pre-ovulatory surge of LH; E_2 also decreases secretion of FSH but the effect is less rapid occurring several hours after administration of E_2 (Beck and Reeves, 1973; Nett *et al.*, 1984; Clarke and Cummins 1984; Clarke *et al.*, 1988; Herring *et al.*, 1991; Mercer *et al.*, 1993; Dhillon *et al.*, 1997; Molter-Gerard *et al.*, 2000).

Unlike the rapid decrease in LH secretion following administration of E_2, previous studies support a genomic mechanism being responsible for the actions of E_2 on the pre-ovulatory surge of LH (Nett *et al.*, 1984; Clarke *et al.*, 1988; Gregg and Nett, 1989; Turzillo *et al.*, 1995a; Kirkpatrick *et al.*, 1998) and the decrease in secretion of FSH (Baratta *et al.*, 2001). As previously mentioned, the most important criterion for establishing a nongenomic action of steroid hormones is that the effect has to be induced within minutes after treatment (Revelli, 1998; Levin, 1999; Simoncini and Genazzani, 2003; Losel *et al.*, 2003). It has been proposed that the decrease in secretion of LH observed within a short time after administration of E_2 appeared to occur too rapidly to be compatible with the classic genomic action of steroid hormones (Nett *et al.*, 1984);

however, the precise time required to effectively suppress secretion of LH is unknown. Because it was not the objective of the studies cited previously, the sampling periods were not designed to evaluate how rapidly LH decreased after treatment with E_2. Moreover, in the majority of previous studies E_2 was administered either subcutaneously or intramuscularly, thus requiring additional time for absorption into the blood, and thus for occurrence of any corresponding response.

Consequently, we employed intravenous delivery of E_2 and the E_2-conjugates to evaluate the biphasic action of E_2 on release of LH from the ovine pituitary gland. We hypothesized that E_2, E_2-BSA, and E_2-PEP would acutely decrease the release of LH. A second hypothesis was that E_2, but not the conjugated forms of E_2 would induce a genomic action on gonadotropin secretion in OVX ewes.

Materials and Methods.

Animals and Experimental Protocol. Between October and January, mature Western-range ewes that had been OVX for at least 2 months received 50 μg of E_2 (n=6), or equimolar concentrations of E_2-BSA (n=5) or E_2-PEP (n=5). Because of the variation in the number of E_2 molecules attached to the original E_2-BSA powder, estimation of E_2-BSA dosage was based on the molecular weight of BSA. Free E_2 was removed from E_2-BSA as described in a previous chapter. To rapidly increase blood levels of E_2 and the conjugates, one third of the dosage was injected intravenously as a priming dose. Beginning immediately after priming, the remaining dosage of each estrogen was infused intravenously over a period of 4 h. All ewes were fitted with indwelling jugular cannulas (B.D. Angiocath) in the right external jugular vein to withdraw blood samples. The left external jugular vein was fitted with Silastic tubing (Cat No 508-004, Laboratory

Tubing). This tubing was connected to an infusion pump for infusion of the estrogens. The infusion device consisted of an auto syringe (Medical Specialties of Australia) with 30-ml plastic syringe connected to an automatic timing device allowing the delivery of 161 µl of physiological saline (0.15 M NaCl) containing 2 nmoles of E_2, E_2-BSA or E_2-PEP every 3:45 min. Blood samples were collected via catheter every 15 min from 4 h before to 5 h after the priming treatments. Additional blood samples were collected at 1 h intervals from 6 to 24 h after priming. Blood was allowed to clot for 1 h at room temperature and then stored at 4 °C overnight. Serum was separated by centrifugation and stored at –20 °C for subsequent quantification of LH and FSH by RIA. The following parameters were examined: 1) Basal LH; defined as the lowest hormone concentration between pulses of LH, 2) Mean LH; defined as the average concentration of LH, 3) Number of pulses of LH; a pulse of LH was defined as a concentration of LH equal or higher than the average concentration of LH plus 2 standard deviations, followed by at least one descending hormone concentration, 4) Interval from priming of treatments to the absence of pulses of LH, and 5) Length of suppression of pulsatile secretion of LH. Basal and mean LH as well as pulses of LH were determined during the 4 h before (pre-infusion period) and the 4 h after (infusion period) priming of treatments. The effect of treatments on the expected pre-ovulatory-like surge of LH was evaluated using five parameters related to the massive release of LH; 1) Mean LH, 2) Amplitude; defined as the highest concentration of LH, 3) Area under the curve during the surge, 4) Length of the pre-ovulatory like surge, and 5) Interval from the priming treatment to the beginning of the massive release of LH. A pre-ovulatory like surge of LH was defined as a concentration of LH equal to or higher than the mean LH (detected during the 4 h prior to

priming) followed by at least 4 ascending hormone concentrations, reaching a maximum concentration of at least 60 ng (which represents the estimation, from several publications, of the highest concentration of LH minus 3 standard deviation during the pre-ovulatory like surge of LH induced by E_2 (Beck and Reeves, 1973; Nett *et al.*, 1984; Clarke and Cummins 1984) and then, decreasing to basal levels. Serum concentrations of FSH during the 4 h prior to (control period) priming of treatments were averaged and compared with the hourly mean concentration of FSH observed after priming.

Radioimmunoassays. Ovine LH (oLH; Niswender *et al.*, 1969) and ovine FSH (oFSH; L'Hermite *et al.*, 1972) were quantified by double antibody radioimmunoassay. NIH-OLH-S24 and NIH-OFSH-S12 were used as standards, for oLH and oFSH, respectively. Triplicate standard curves were included in each assay and all serum samples were analyzed in duplicate at 50 µl sample/tube for oLH, and 200 µl sample/tube for oFSH. Hormones were radiolabeled with ^{125}I by the chloramine T procedure (Niswender *et al.*, 1975). Radioiodinated oLH and oFSH were separated from free ^{125}I on a 10 ml G-25-sephadex chromatography column eluted with PBS gel.

Statistical Analysis. Data were subjected to analysis of variance (ANOVA) using the general linear model of SAS (1987). Basal LH, mean LH, and number of pulses of LH as well as changes in secretion of FSH were subjected to repeated measures analysis. Number of pulses of LH was subjected to Arc Sine transformation. Sources of variation included in the model were treatment, period, and the interaction of treatment by period. Ewe nested in treatment was used as the error term for treatment effect. Parameters related to intervals and parameters evaluated during the pre-ovulatory-like surge of LH

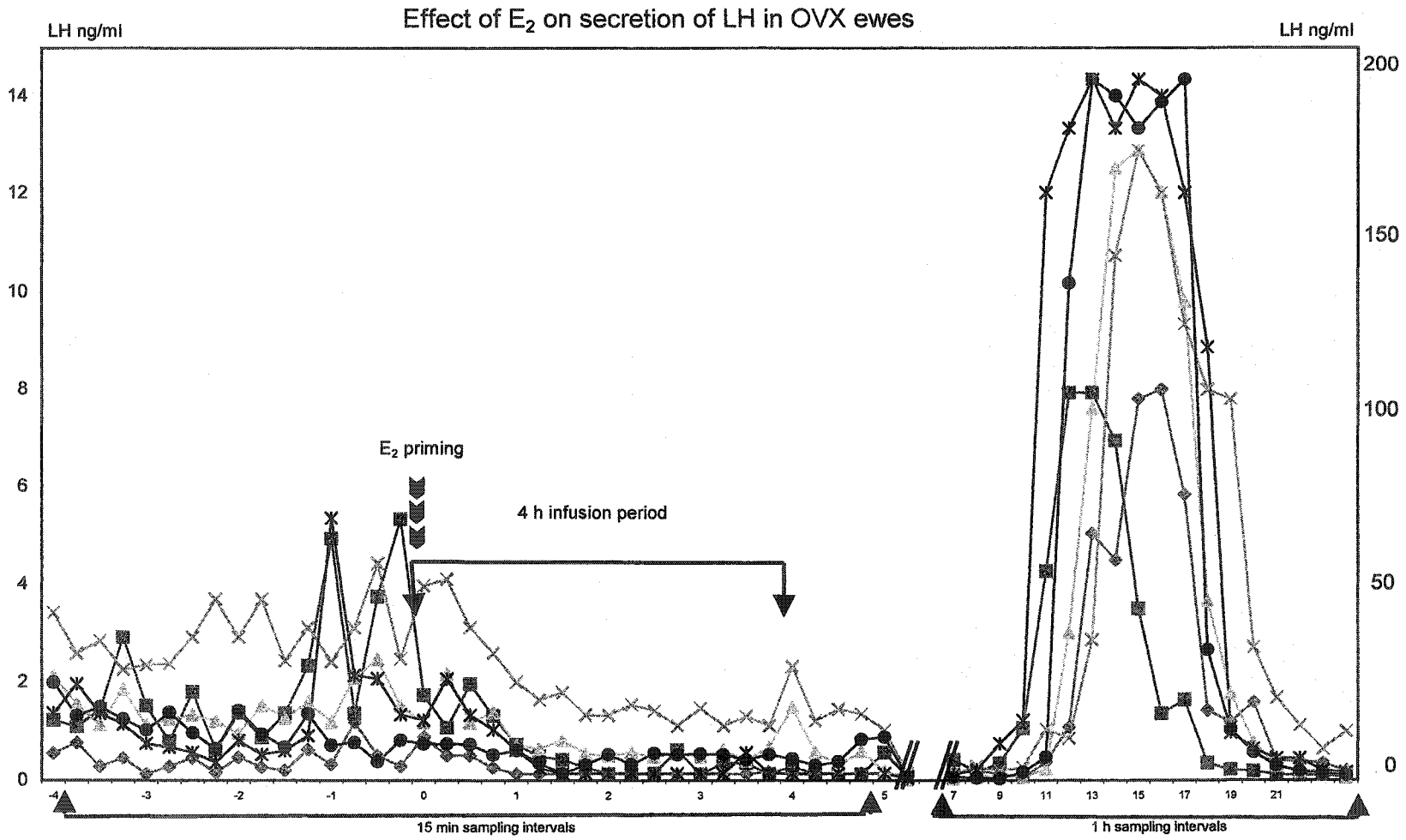
were analyzed in a completely randomized design. When differences among treatment means were detected, they were separated using LSD adjusted by Tukey's procedure.

Results

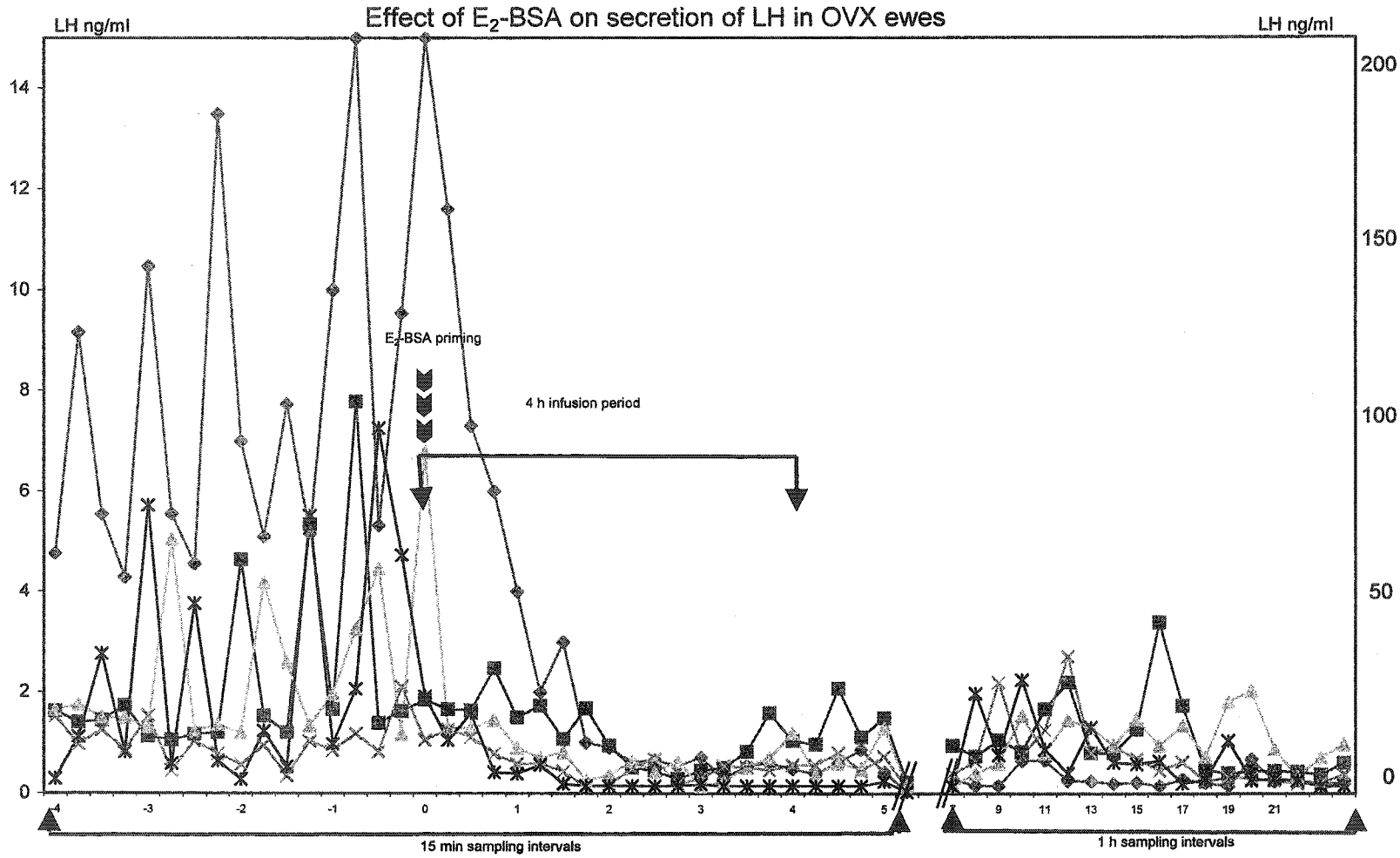
Radioimmunoassays. Intra-assay coefficient of variation for LH (n=5) and FSH (n=6) averaged 5% and 6%, respectively. Inter-assay coefficient of variation for LH (n=5) and FSH (n=6) were 11% a 13%, respectively. The minimum detectable dosage of LH and FSH averaged 26 pg and 1.9 ng, respectively.

Effect of E_2, E_2-BSA, and E_2-PEP on Gonadotropin Secretion. Changes in the secretory profile of LH by treatment with E_2, E_2-BSA, and E_2-PEP are depicted in Figures 5.1, 5.2, and 5,3, respectively. Before the priming treatment, basal LH, mean LH, as well as number of pulses of LH were similar ($p > 0.2$) among ewes treated either with E_2, E_2-BSA or E_2-PEP, and there was not a significant ($p > 0.2$) interaction between treatment and period in any of the variables examined. Treatment with E_2, or either conjugated form of E_2, decreased basal LH (mean ± standard error; from 1.22 ng/ml ± 0.28 to 0.52ng/ml ± 0.10; $p < 0.05$; Figure 5.4), mean LH (from 2.12 ng/ml ± 0.51 to 0.92 ng/ml ± 0.23; $p < 0.05$; Figure 5.5), and number of pulses of LH (from 3.94 pulses/4h ± 0.25 to 0.88 pulses/4h ± 0.21; $p < 0.01$; Figure 5.6) compared to the pre-treatment period. Pulses of LH were rapidly abolished after priming with E_2, E_2-BSA or E_2-PEP (19 min ± 5; $p > 0.02$; Figure 5.7). After the initial suppression, no pulsatile activity was detected for the next 245 ± 5 min regardless of treatment ($p > 0.02$; Figure 5.8).

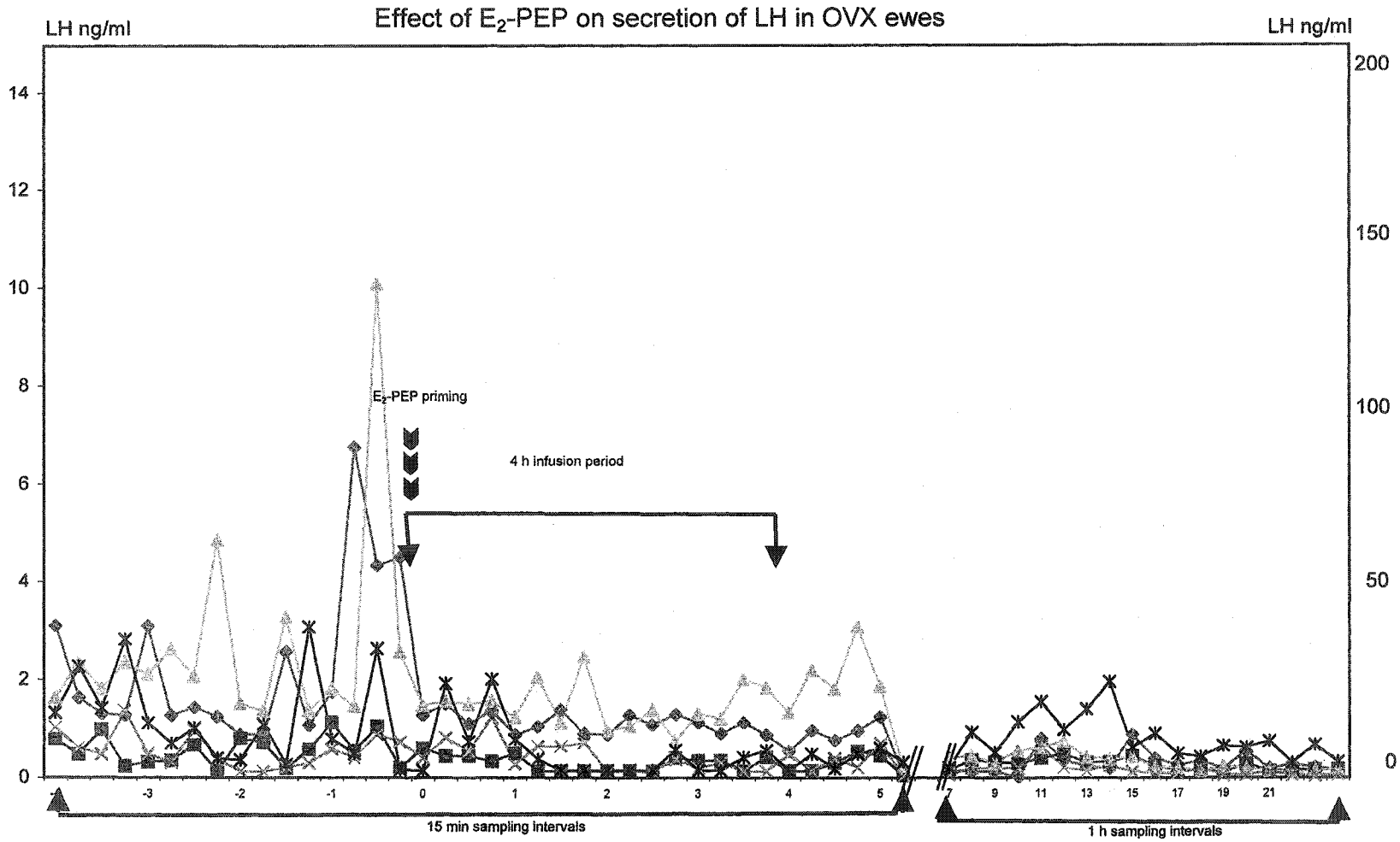
Regarding the effect of treatment on the induction of a pre-ovulatory-like surge of LH, only E_2 induced a massive release of LH, starting 590 ± 32 min after E_2 priming.



Fifure 5.1. Six OVX ewes were infused with E_2 for 4 h. The scale on the left depicts LH levels from blood samples collected every 15 min from 4 h before to 5 h after priming with E_2. The scale on the right depicts LH levels during the masive surge of LH. Treatment with E_2 induced a rapid decrease in secretion of LH, followed hours later for the characteristica pre-ovulatory like surge of LH.



Fifure 5.2. Five OVX ewes were infused with E_2-BSA for 4 h.The scale on the left depicts LH levels from blood samples collected every 15 min from 4 h before to 5 h after priming with E_2-BSA. The scale on the right depicts LH levels during the expected masive surge of LH. Treatment with E_2-BSA induced a rapid decrease in secretion of LH;however E_2-BSA did not induce a pre-ovulatory like surge of LH.



Fifure 5.3. Five OVX ewes were infused with E_2-PEP for 4 h.The scale on the left depicts LH levels from blood samples collected every 15 min from 4 h before to 5 h after priming with E_2-PEP. The scale on the right depicts LH levels during the expected masive surge of LH. Treatment with E_2-PEP induced a rapid decrease in secretion of LH;however E_2-PEP did not induce a pre-ovulatory like surge of LH.

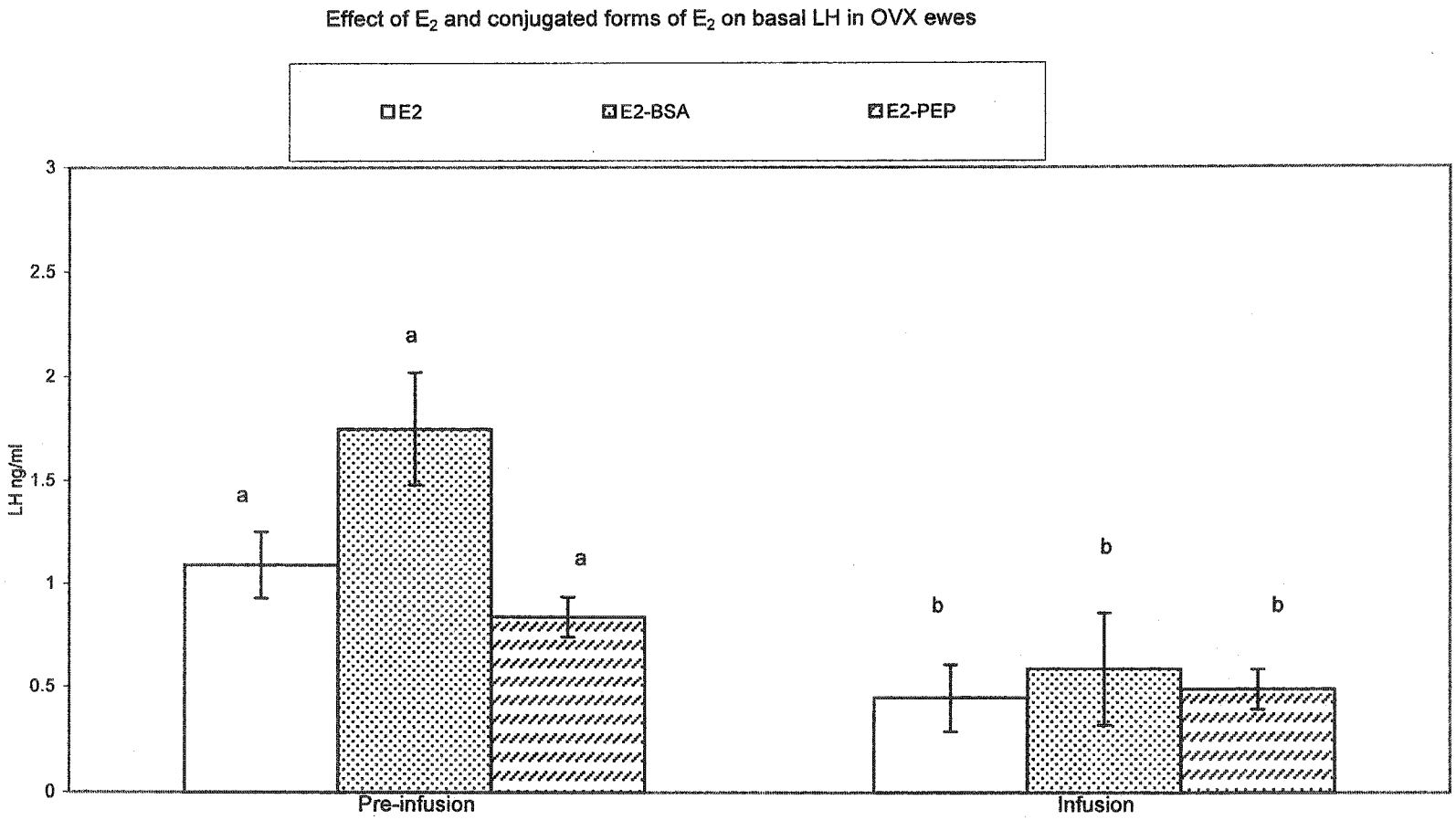


Figure 5.4. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. Basal LH was estimated from blood samples collected every 15 min during 4 h before and 4h after the beginning of infusion. Comparisons were made within treatments; $P < 0.05$. Mean ± standard error.

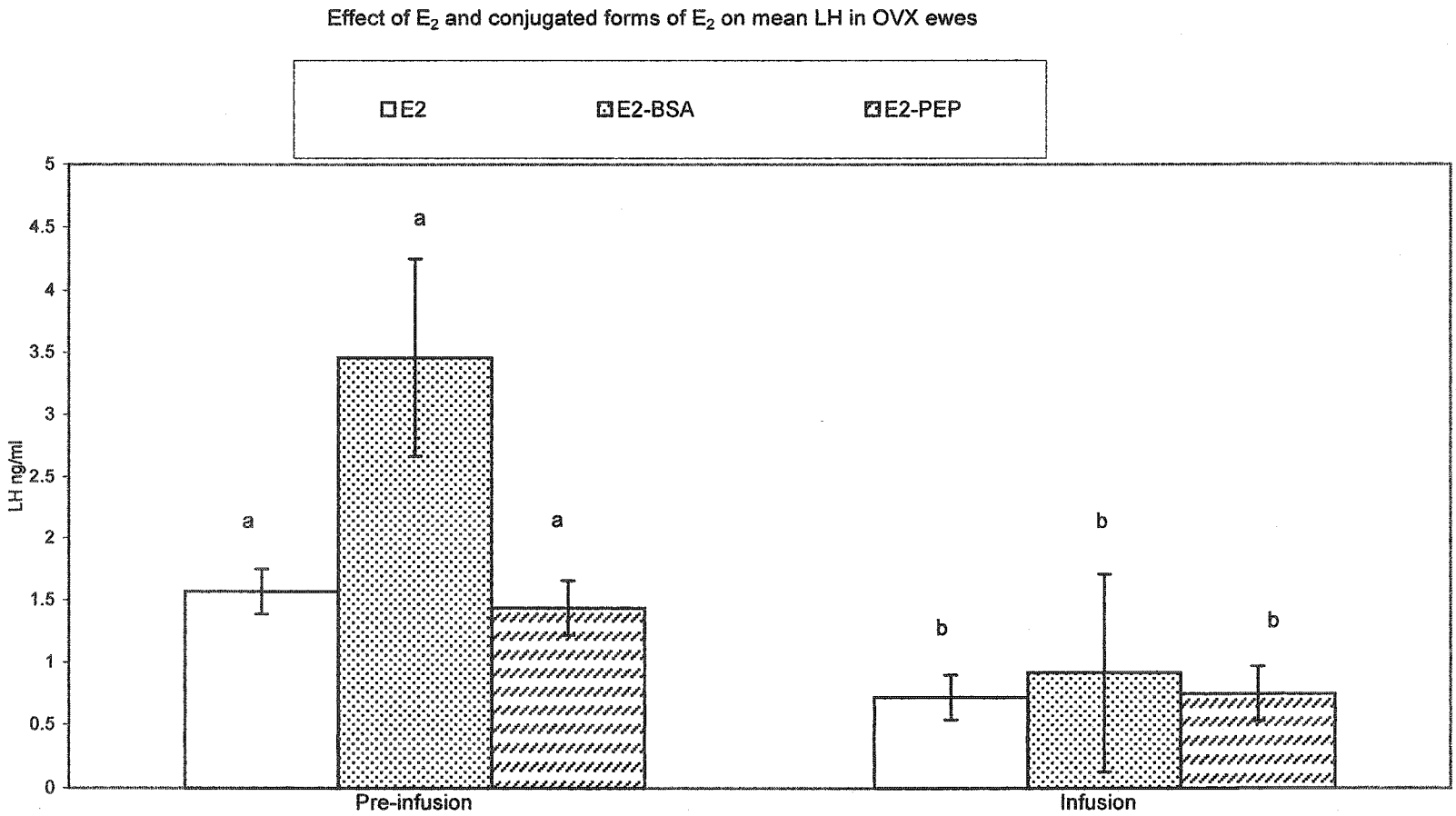


Figure 5.5. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. Media LH was estimated from blood samples collected every 15 min during 4 h before and 4h after the beginning of infusion. Comaparions were made within treatments; $P < 0.05$. Mean ± standard error.

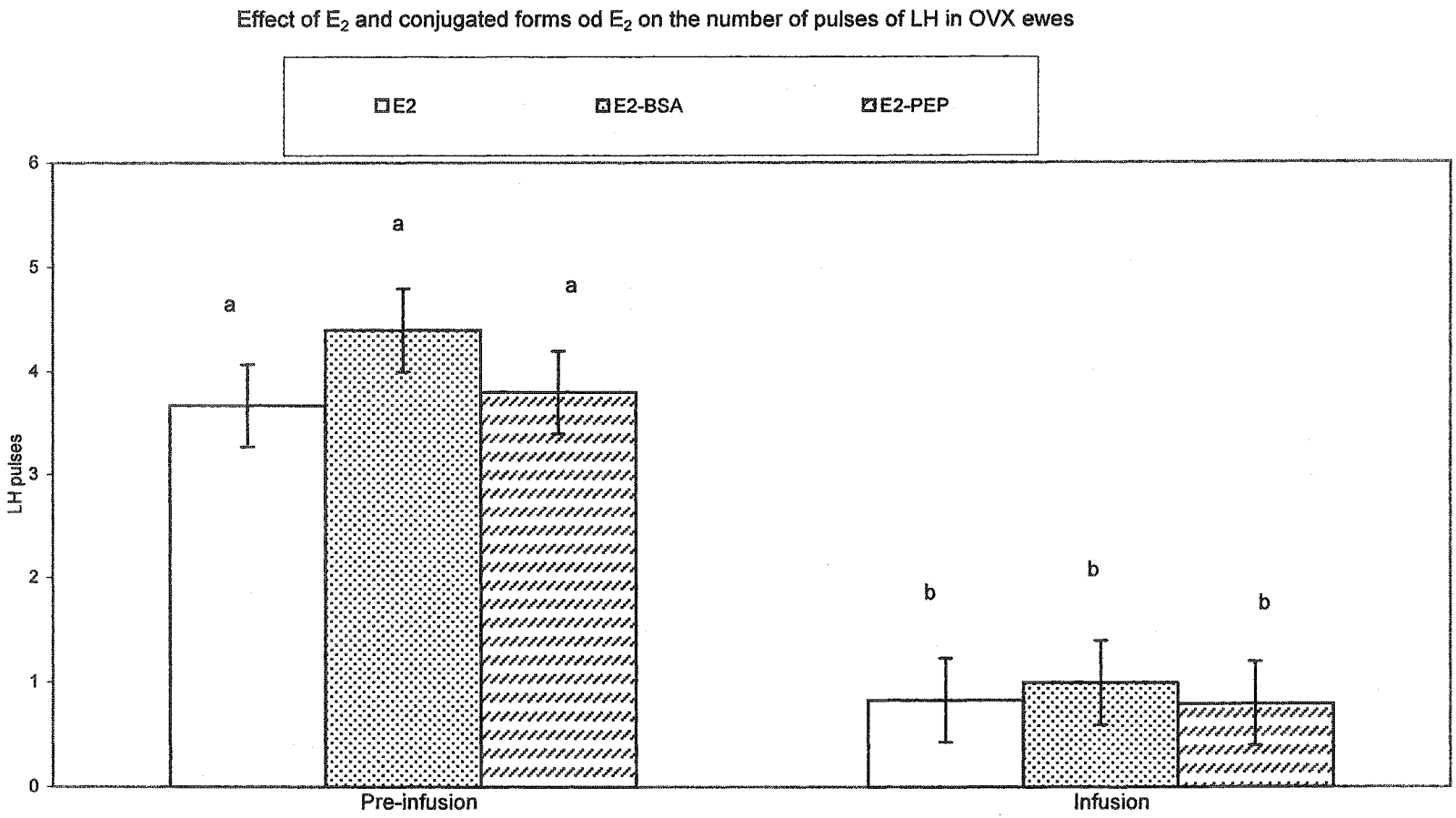


Figure 5.6. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. LH pulses were estimated from blood samples collected every 15 min during 4 h before and 4h after the beginning of infusion. Comparisons were made within treatments; mean ±standard errors; $P < 0.01$.

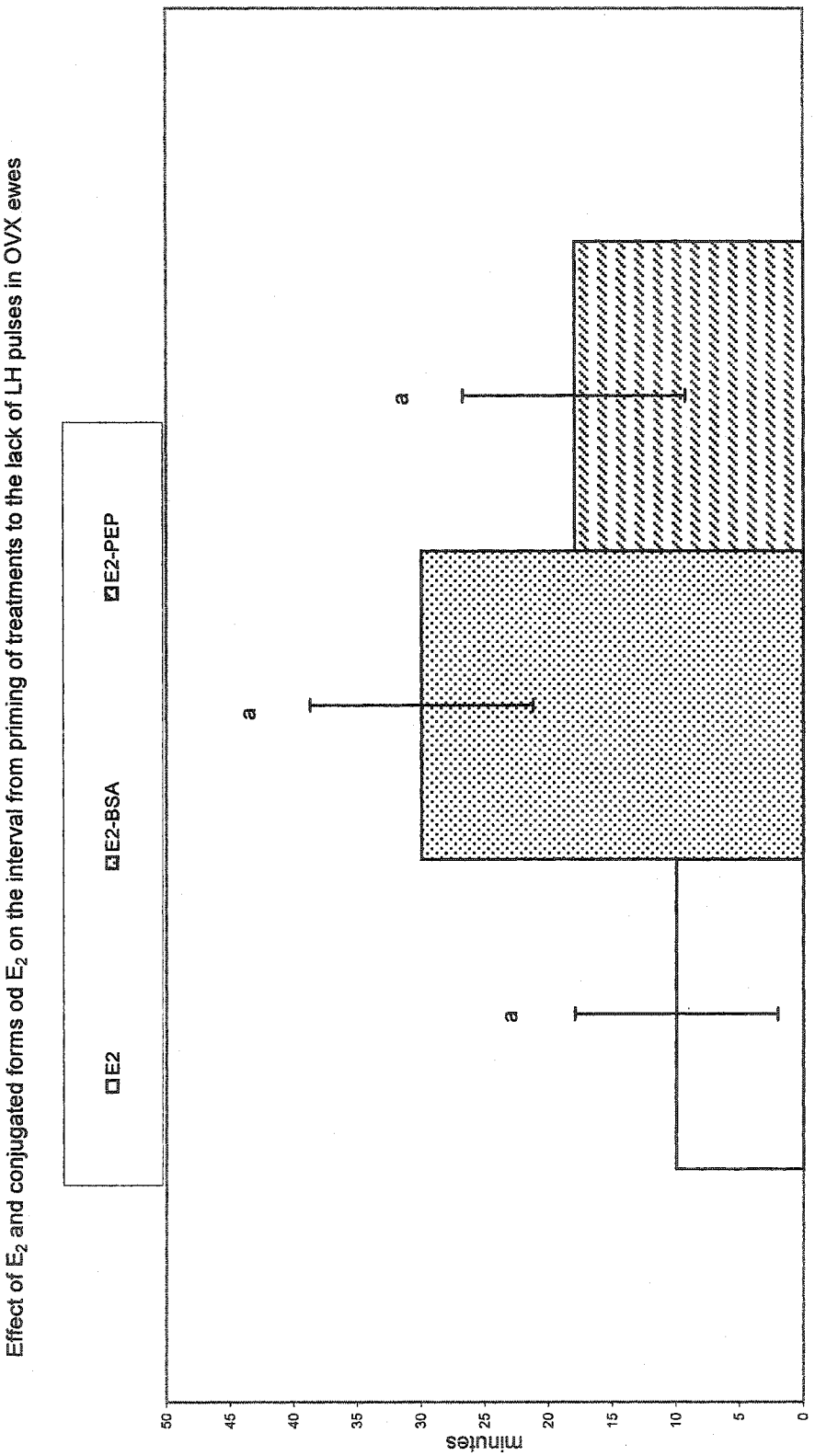


Figure 5.7. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. Mean ± standard error; P > 0.1.

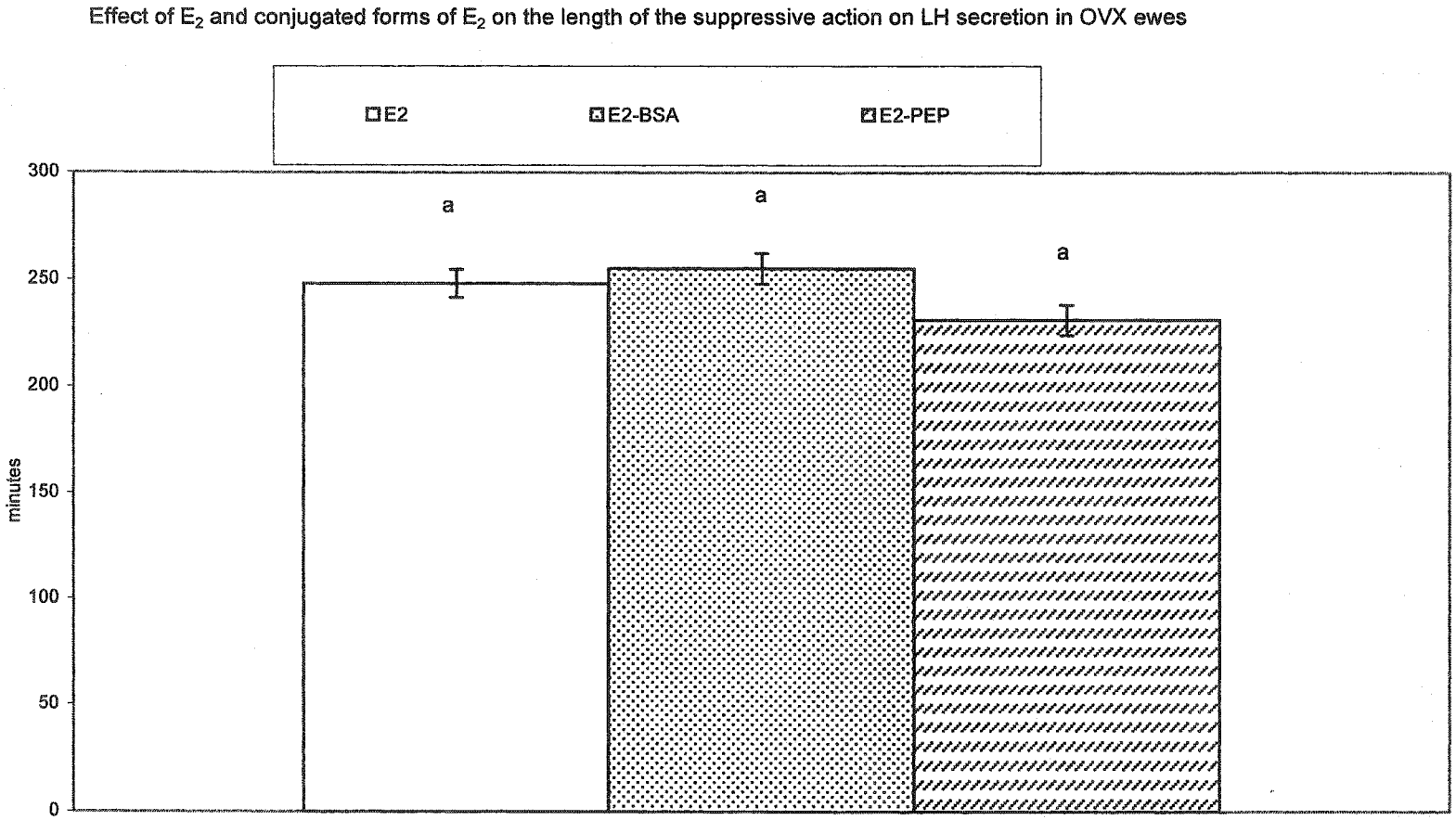


Figure 5.8. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. Suppressive period was the interval without pulses of LH. Mean ± standard error; $P > 0.1$.

The duration of the surge was 775 ± 72 min. Although, E_2-BSA and E_2-PEP did not induce a massive release of LH, mean, amplitude, and area under the curve of LH during the expected pre-ovulatory like surge were estimated during the time interval when an LH surge should have occurred and compared with the surge observed in E_2-treated ewes. The massive release of LH induced by E_2 was characterized by a mean LH of 91.1 ± 8.19 ng/ml, an amplitude of 175.8 ± 12 ng/ml, and an area under the curve of LH of 998.34 ± 122 arbitrary units. All these parameters were higher ($p < 0.001$; Figures 5.9 to 5.11) in E_2 treated ewes than those observed during same interval in E_2-BSA and E_2-PEP treated ewes. Although conjugated forms of E_2 did not induce a pre-ovulatory like surge of LH, the mean LH detected during the pre-ovulatory like surge of LH was higher than that detected during pre-infusion period (8.99 ± 1.28 versus 2.45 ± 1.28 ng/ml; $p < 0.01$).

Before priming with the estrogens, serum concentrations of FSH were similar (490 ± 32.28 ng/ml $p > 0.2$) among ewes treated either with E_2, E_2-BSA or E_2-PEP. Treatment with E_2 decreased ($p > 0.1$) FSH secretion (Figure 5.12) at 4 h (503 ± 30.35 vs. 379 ± 30.2 ng/ml) after priming of E_2 and FSH remained low during the next 8 h (503 ± 30.35 vs. 327 ± 30.05 ng/ml). Neither E_2-BSA (Figure 5.13) nor E_2-PEP (Figure 5.14) affected the secretory pattern of FSH ($p > 0.1$).

Discussion. The priming/infusion system used to intravenously supply E_2 proved to efficiently induce both the acute and the delayed effects of E_2 on gonadotropin secretion. As in our study, pharmacological doses of E_2 have been used successfully to induce the biphasic modulation in secretion of LH in OVX ewes (Nett *et al.*, 1984;

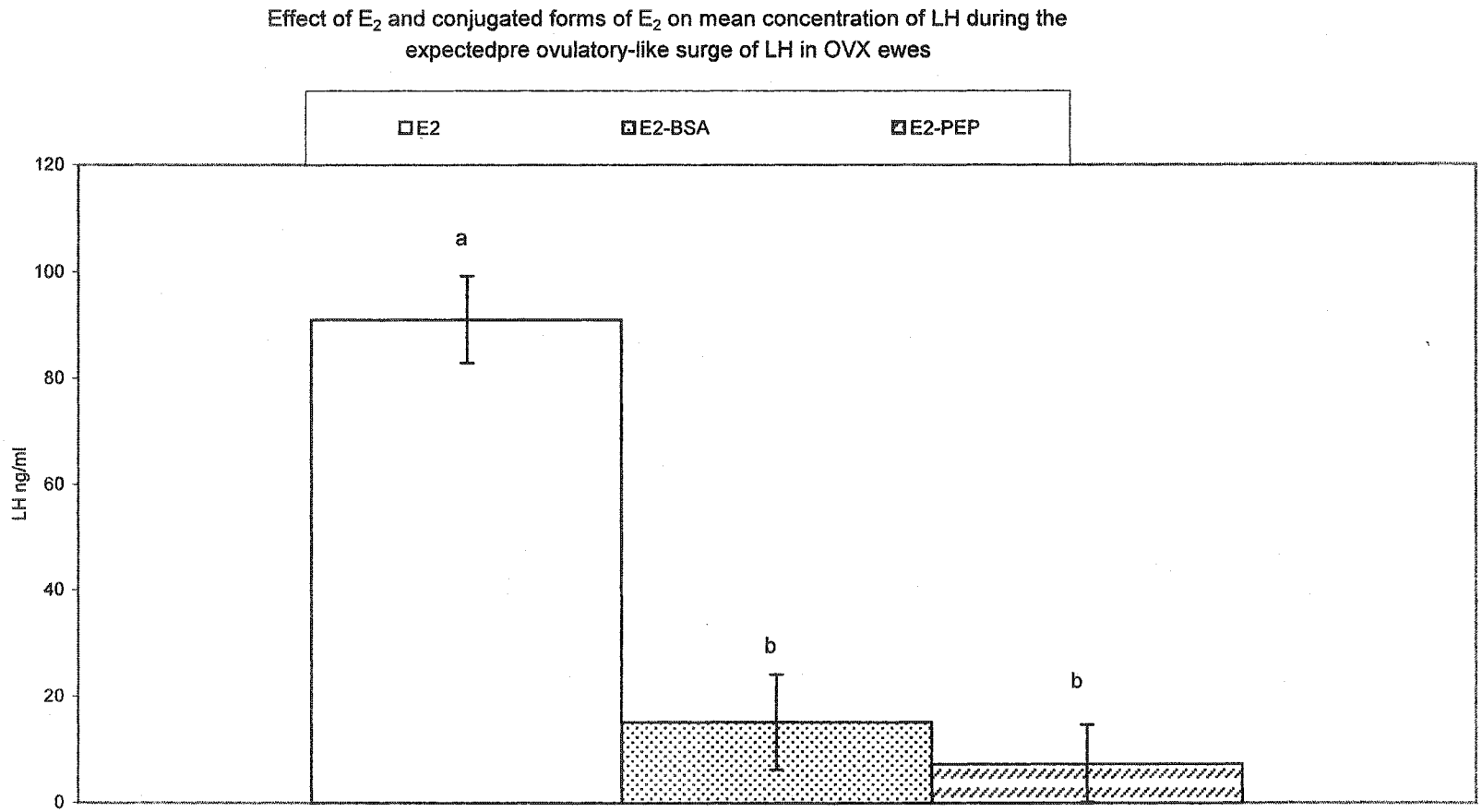


Figure 5.9. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. A pre-ovulatory like surge of LH was detected only in E_2 treated ewes. Mean ± standard error; $P < 0.01$.

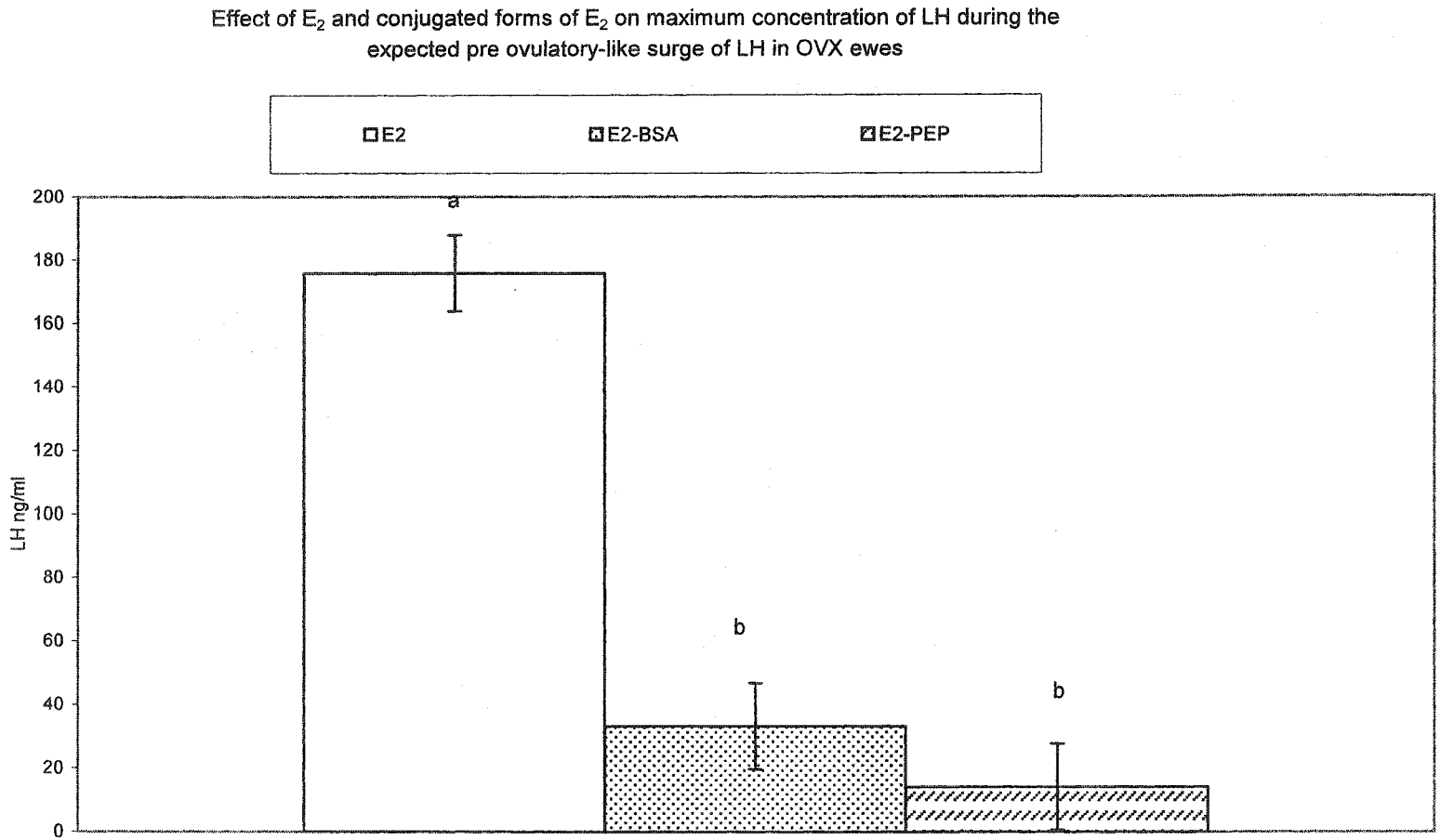


Figure 5.10. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. A pre ovulatory-like surge of LH was detected only in E_2 treated ewes. Mean ± standard error; $P < 0.01$.

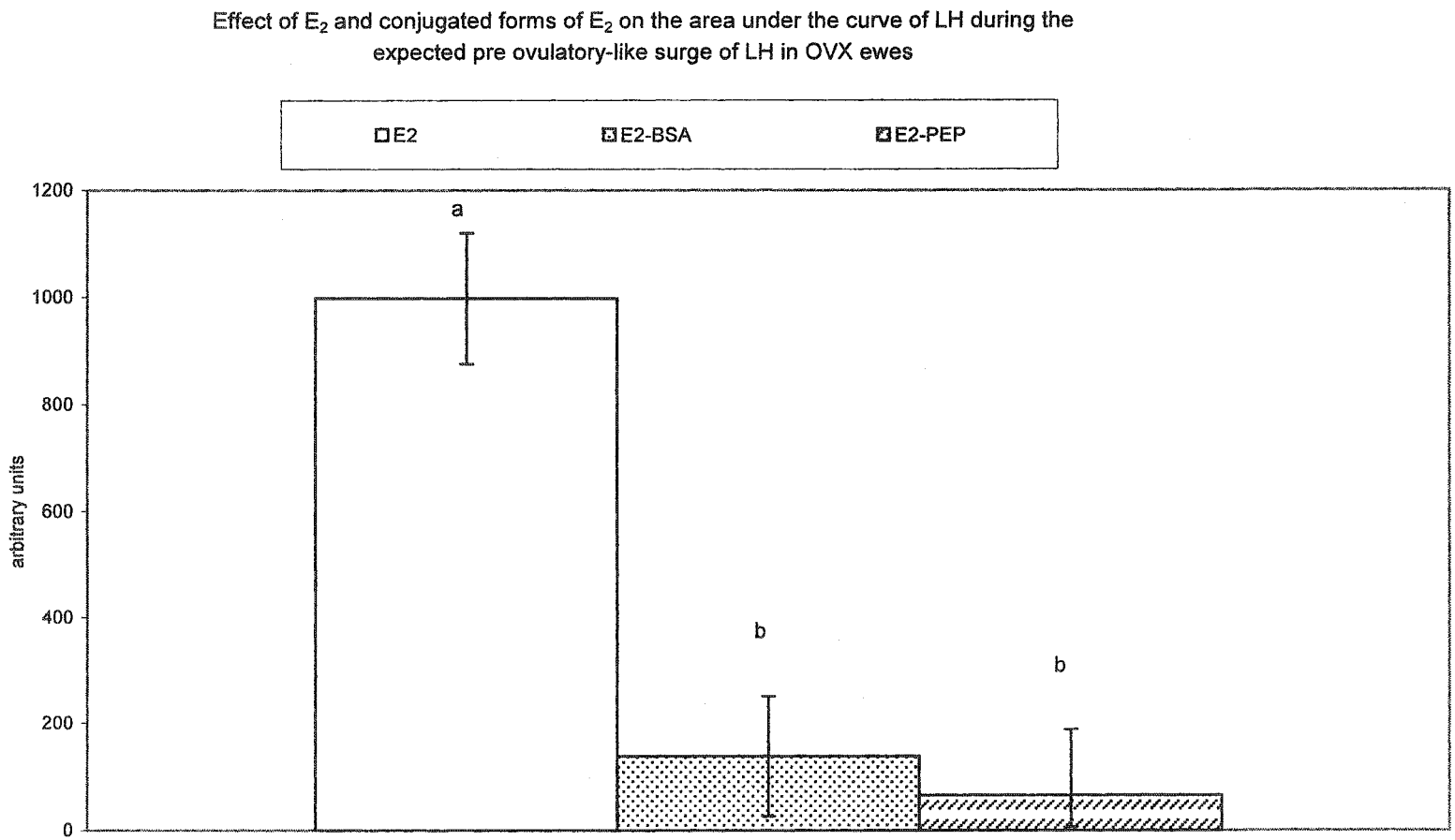


Figure 5.11. OVX ewes were infused with E_2 (n=6), E_2-BSA (n=5), or E_2-PEP (n=5) for 4 h. A pre ovulatory-like surge of LH was detected only in E2 treated ewes. Mean ± standard error; $P < 0.01$.

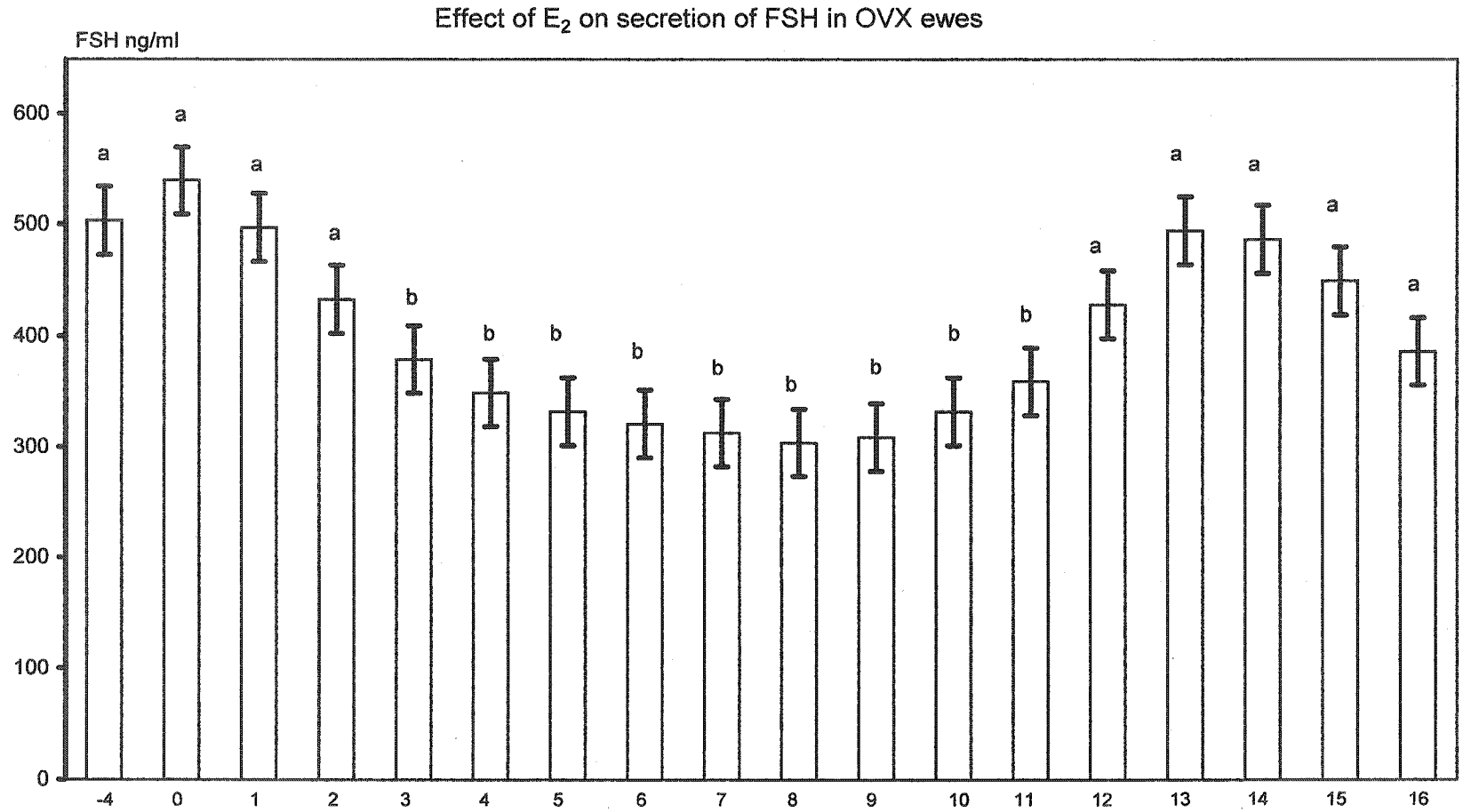


Figure 5.12. Six OVX received E_2 infusion for 4 h. -4 = pretreatment, average FSH from -4 to 0 hr; 1 to 16 = hourly average FSH. Mean ± standard error; Comparisons to pretreatment vs. hourly period; $P < 0.01$.

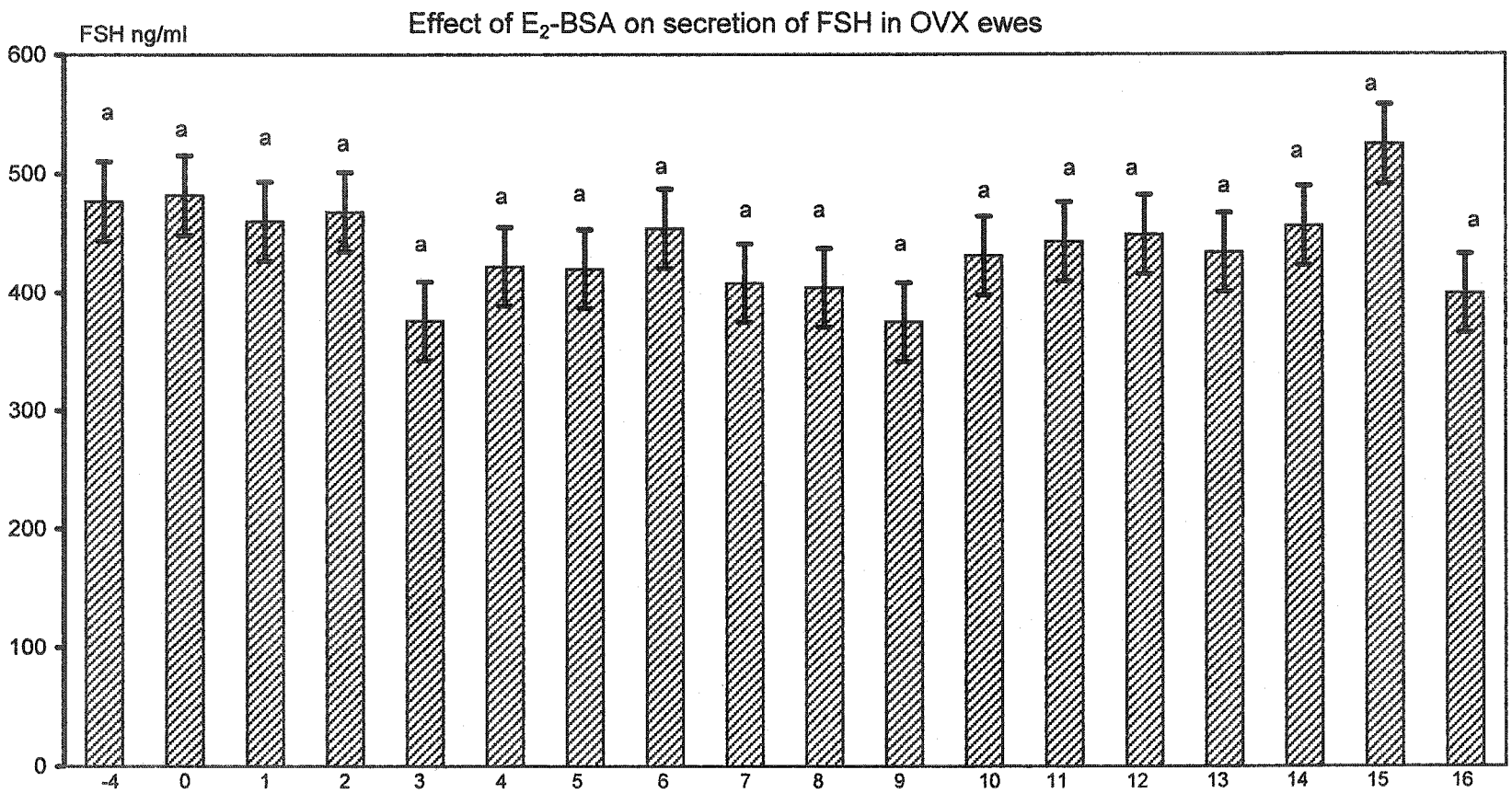


Figure 5.13. Five OVX received E_2-BSA infusion for 4 h. -4 = pre-treatment, average FSH from -4 to 0 hr; 1 to 17 = hourly average FSH. Mean ± standard error; Comparisons of pretreatment vs. hourly period.

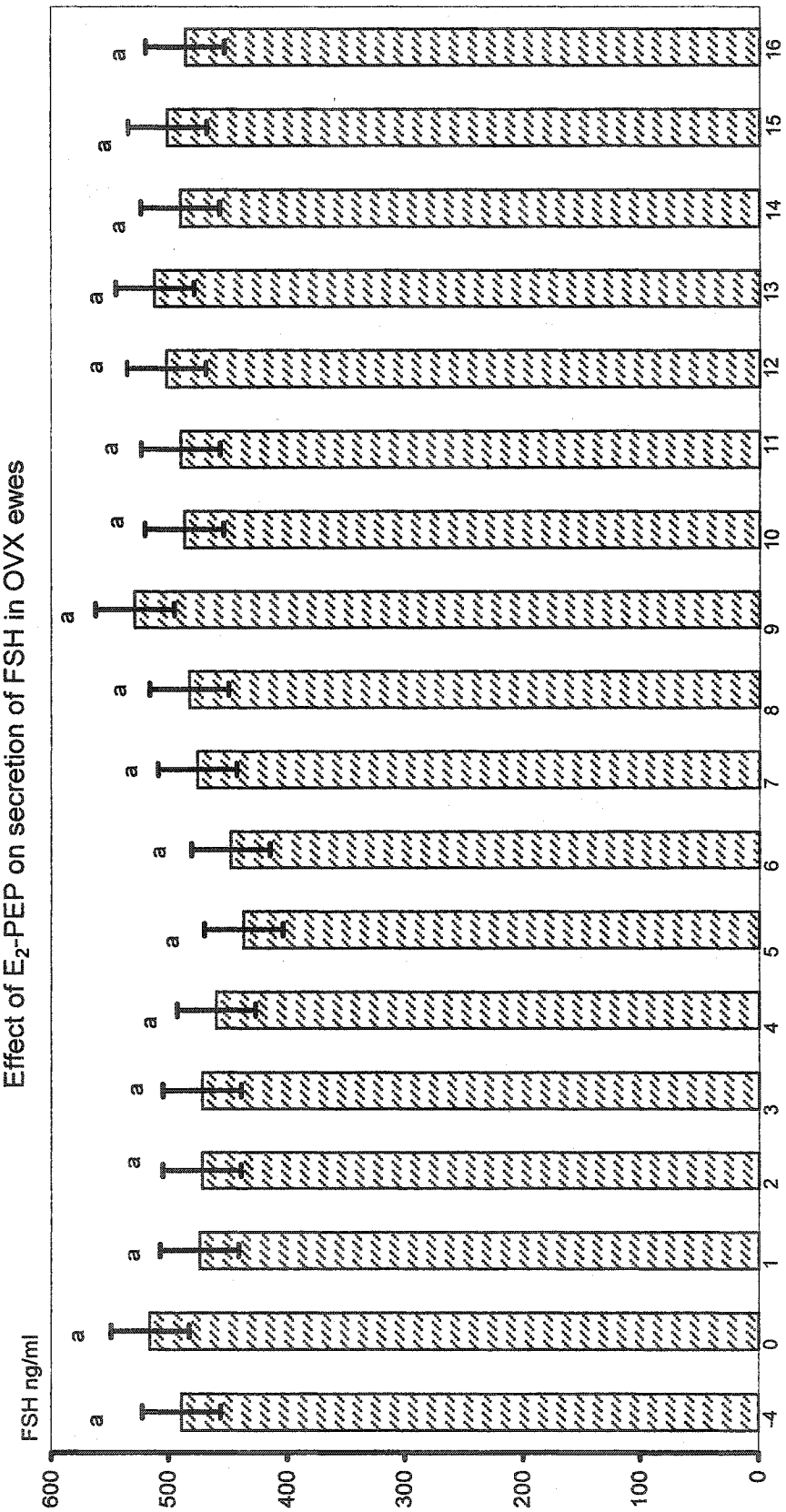


Figure 5.14. Five OVX received E_2-PEP infusion for 4 h. -4 = pre-treatment, average FSH from -4 to 0 hr; 1 to 17 = hourly average FSH. Mean ± standard error; Comparisons of pretreatment vs. hourly period.

Clarke and Cummins, 1985; Mercer, *et al.*, 1993; Molter-Gerard *et al.*, 2000) resembling the pre-ovulatory surge of LH observed in intact ewes (Karsch *et al.*, 1980).

In OVX ewes, E_2 acutely suppressed pulsatile secretion of LH. After the initial suppression, no pulses of LH were observed for the remainder of the infusion period. The acute suppression of the pulsatile release of LH corroborates our *in vitro* finding that E_2 decreased pituitary sensitivity to GnRH, and agrees with previous studies where, by using OVX/HPD ewes maintained with GnRH pulses, a pituitary loci as the site for the negative action of E_2 was postulated (Clarke *et al.*, 1988; Gregg and Nett, 1989; Mercer, *et al.*, 1993). In earlier studies, a decrease in secretion of LH was detected within an hour after E_2 treatment in OVX and OVX/HPD ewes (Nett *et al.*, 1984; Gregg and Nett, 1989). In our study, the frequent blood sampling, together with the priming treatments, allowed a more accurate detection of the beginning of negative feedback by E_2. In our hands, LH pulses were suppressed within 20 min after the beginning the infusion of E_2. Because the half-life of LH in ewes is about 20 min (Akbar *et al.*, 1974), we did not expect to detect a suppression of pulses earlier than this. Thus, it appears that the mechanism responsible for suppression of LH pulses was activated almost immediately after administration of the priming of the dose of estrogens. Therefore, the sudden suppression of LH secretion is not compatible with the time necessary for a genomic action to occur. For example, in ewes, *de novo* synthesis of GnRH receptors required a minimum of 4 h (Nett *et al.*, 1984; Gregg *et al.*, 1989; Turzillo *et al.*, 1998a,b). Although the hypothalamus is a recognized target for both stimulatory and inhibitory actions of E_2 (Legan *et al.*, 1977; Karsch *et al.*, 1987; Karsch *et al.*, 1993; Moenter *et al.*, 2003), apparently the acute suppression in secretion of LH induced by E_2 is not mediated by a direct effect at level of the

hypothalamus. This is supported by the fact that frequency of pulses of GnRH, quantified in hyposphyseal portal blood, remained without changes during the acute negative feedback of E_2 on secretion of LH (Clarke and Cummins *et al.*, 1985).

E_2-BSA and E_2-PEP were as effective as E_2 in acutely suppressing pulsatile secretion of LH and abolishing pulses of LH during the infusion of treatments. Although the ability of the commercially available E_2-BSA to mimic acute actions E_2 has been widely demonstrated *in vitro* (Prevot *et al.*, 1999; Kim *et al.*, 1999; Razandi *et al.*, 1999; Razandi *et al.*, 2000a; Russell *et al.*, 2000; Hisamoto *et al.*, 2001b; Wade *et al.*, 2001; Santos *et al.*, 2002; Wong *et al.*, 2003; Chen *et al.*, 2003), this is the first report that E_2-BSA and a novel synthetic E_2-PEP mimic the acute action of E_2 in an *in vivo* model. Moreover, E_2, but not E_2-BSA or E_2-PEP, induced the characteristic pre-ovulatory like surge of LH, beginning ~10 h after the priming dose. Timing and magnitude of the LH surge is consistent with observations reported by others (Beck and Reeves, 1973; Nett *et al.*, 1984; Clarke and Cummins 1984; Clarke *et al.*, 1988; Herring *et al.*, 1991; Mercer *et al.*, 1993; Dhillon *et al.*, 1997; Molter-Gerard *et al.*, 2000). It has been well documented that the pre-ovulatory surge of LH observed after administration of E_2 is due to at least 2 components, first an increase in the number of GnRH receptors in the pituitary gland (Nett *et al.*, 1984; Emons *et al.*, 1988; Clarke *et al.*, 1988; Gregg and Nett, 1989; Gregg *et al.*, 1990; Laws *et al.*, 1990; Turzillo *et al.*, 1995a; Quiñones-Jenab *et al.*, 1996; Kirkpatrick *et al.*, 1998a,b) and second, a pre-ovulatory surge of GnRH from hypothalamus (Moenter *et al.*, 1990; Moenter *et al.*, 1993; Evans *et al.*, 1994; Caraty *et al.*, 1998). The fact that conjugated forms of E_2 did not induce a pre-ovulatory like surge of LH indicates that the attachment of a carrier molecule as BSA or a small peptide to E_2

impaired the gene expression resulting in the massive release of LH. It is thought that the carrier molecule attached to E_2 prevents or delays internalization of E_2. Indeed, E_2-BSA, distinct from E_2, did not stimulate reporter activity in tumoral and non-tumoral cell lines transfected with an ERE-luciferase reporter construct (Watters *et al.*, 1997; Razandi *et al.*, 1999; Razandi *et al.*, 2000b; Wade *et al.*, 2001), even when reporter activity was evaluated up to 8 h after treatment (Razandi *et al.*, 2000b; Wade *et al.*, 2001). Although internalization rate of conjugated forms of E_2 have not been evaluated, the previous results have been interpreted as evidence that E_2-BSA does not enter to the cell to bind to nuclear ER, nor does it dissociate into E_2 and BSA components. An alternative or complementary interpretation would be that the carrier molecule interferes with the precise coupling among E_2-ER, DNA sequences, and co-factors required for gene expression.

E_2 but not its conjugated forms decreased secretion of FSH. The decrease in secretion of FSH was first noted ~4 h after E_2 priming and the concentration of FSH remained low during the next 8 h. Therefore, the time-frame for the delayed decrease in FSH is consistent with the idea of a genomic mechanism underlying the negative feedback of E_2 in secretion of FSH. Similar negative feedback effects of E_2 on secretion of FSH have been reported previously (Reeves *et al.*, 1974; Mercer *et al.*, 1989; Mercer *et al.*, 1993; Molter-Gerard *et al.*, 2000). A direct effect of E_2 on secretion of FSH at level of the pituitary gland has been demonstrated using OVX/HPD ewes maintained with GnRH pulses (Mercer *et al.*, 1989; Mercer *et al.*, 1993; Turzillo *et al.*, 1998a) and in cultured ovine pituitary cells (Miller *et al.*, 1977; Huang and Miller, 1980). In both *in vivo* and *in vitro* studies, the decrease in secretion of FSH was accompanied by a decrease

in levels of FSHβ mRNA in the pituitary gland (Alexander and Miller, 1982; Mercer *et al.*, 1989; Mercer *et al.*, 1993; Shupnik, 1996; Turzillo *et al.*, 1998a; Molter-Gerard *et al.*, 2000; Baratta *et al.*, 2001). Apparently, the negative action of E_2 on FSHß gene expression in sheep is mediated by inhibition of pituitary β_B (Activin B) gene expression (Baratta *et al.*, 2001). The fact that conjugated forms of E_2 did not induce the delayed suppression of secretion of FSH observed with E_2, reaffirm the observation that conjugated forms of E_2 are not capable of mimicking the genomic actions induced by E_2, further supporting the concept of a nongenomic mechanism underlying the acute suppression of LH.

The mechanism by which E_2 acutely suppressed GnRH-induced release of LH has yet to be elucidated. As discussed in the introductory chapter, E_2 can influence a variety of signaling pathways, several of which are reported to be involved in the release of LH induced by GnRH. Specifically, E_2 has been implicated in uncoupling specific GPCRs from their effector system (Nunemura *et al.*, 1989), perhaps by stabilizing the association of the heterotrimeric G-protein subunits (Maus *et al.*, 1990). Likewise, E_2 is able to stimulate (Lieberherr *et al.*, 1993; Benten *et al.*, 2001; Guo *et al.*, 2002) or inhibit (Nakajima *et al.*, 1995; Mermelstein *et al.*, 1996; Ruehlmann *et al.*, 1998) L-type Ca^{2+} currents in a cell dependent manner. Another possible candidate involved in the inhibition of GnRH-induced secretion of LH caused by E_2 is NOS. In rat pituitary cells NO decreased GnRH-induced release of LH (Ceccatelli *et al.*, 1993); whereas in the hypothalamus E_2 enhances nNOS mRNA (Ceccatelli *et al.*, 1996) and acutely increases NO production, resulting in GnRH release (Prevot *et al.*, 1999). However, a direct action of E_2 on NOS activity at level of the pituitary gland has not been documented.

At this point it is important to speculate about a possible model by which E_2 induces both, the acute suppression and the delayed stimulation in secretion of LH in the context of ERs subtypes (ERα and ERβ) and their putative location (plasma membrane versus cytoplasmatic/nuclear) in the cell. In both *in vivo* and *in vitro* studies, conjugated forms of E_2 were capable of mimicking the acute suppression in secretion of LH, but not the delayed induction of a pre-ovulatory surge of LH or the delay suppression in secretion of FSH induced by E_2 in OVX ewes. If, as suggested by our data and proposed by others, conjugated forms of E_2 are acting on the cell surface, it is plausible to speculate that ERs located on the plasma membrane mediate the acute suppression of LH induced by E_2. Moreover, the identity of this plasma membrane ER was suggested by the ability of a selective ERα agonist to mimic the acute suppression of LH induced by E_2 in ovine pituitary cells. Therefore, the ER subtype as well as the cellular location of the receptor, appear to be important determinants in the secretory response of pituitary cells to E_2.

As discussed in the introductory chapter, E_2 modulation of signaling pathways initiated in the plasma membrane is evolving as an important component in the regulation of E_2-target genes. The general idea is that E_2 binds to ERs putatively located on the plasma membrane, activates second messenger signals resulting in recruitment of transcription factors, ultimately enhancing the transcriptional activation rate induced by E_2-ER complex. Therefore, both nongenomic and genomic mechanisms work in concert to induce the same cellular response. In this regard, our model presents a distinctive characteristic consisting in two effects of E_2, a acute, nongenomic inhibitory action and a delayed, genomic stimulatory one; however, if the pre-ovulatory surge of LH is considered as the final physiological response, these apparent opposite actions will result

in a more robust response. In this scenario the binding of E_2 to plasma membrane ER (probably ERα) in the pituitary gland, would modulate ion channels and/or second messenger signals blocking GnRH-induced secretion of LH. Simultaneously, E_2 binds to intracellular ERs initiating the synthesis of mRNA for GnRH receptors and maybe for LHβ subunit, which in turn will increase the pituitary content of LH. Both genomic actions of E_2 would be enhanced by recruitment of transcription factors through activation of second messengers induced by ER located on the plasma membrane; however, as mentioned above, the E_2-ERα complexes on the plasma membrane maintain the insensitivity of gonadotrops to the secretagogue actions of GnRH, suppressing pulsatile secretion of LH, further contributing to build up a robust surge of LH in response to the E_2-induced massive release of GnRH. If as we proposed, conjugated forms of E_2 bound only to ERs located on the plasma membrane, an increase in the number of GnRH receptors would not be expected. The lack of an increase in GnRH receptors number may be part of the explanation why conjugated forms of E_2 induced a slight increase in serum concentrations of LH during the expected pre-ovulatory surge. It is also speculated that neither E_2-BSA nor E_2-PEP induced a massive release of GnRH from hypothalamus. If the last statement were not correct, the induction of a massive release of GnRH by conjugated E_2 would be enough to induce a pre-ovulatory like surge of LH. It is known that exogenous GnRH is able to induce a pre-ovulatory like surge of LH in absence of the stimulatory action of E_2 on the number of GnRH receptors (Nett *et al.*, 1984). Whether or not conjugated forms of E_2 are able to cross the blood brain barrier is unknown; however an impermeable blood brain barrier for both of these conjugates is consistent with their lack of effect in the hypothalamus. Alternatively, if conjugated E_2

were able to cross the blood brain barrier, the slight increase in secretion of LH detected during the time of the expected pre-ovulatory surge would be interpreted as the result of impaired and erratic secretion of GnRH as a consequence of a poor or absent actions of conjugated E_2 on synthesis and or secretion of GnRH.

There are several physiological stages during the reproductive life of domestic animals characterized by a switch from a negative to a positive sensitivity of the hypothalamus-pituitary axis to the effects of E_2. These endocrine scenarios are the transition to puberty, the transition to breeding season, and the return of cyclic ovarian activity after parturition. It is tempting to speculate that the nature of this switch may be dependent upon the context of ERs subtypes present during the differing physiological state and their location in the cell (i.e. membrane or nuclear).

In conclusion, the acute inhibition of secretion of LH induced by E_2 in OVX ewes is not compatible with the classic genomic mechanism by which steroid hormones modulate cellular function. An acute, nongenomic action as the mechanism underlying the sudden suppression in secretion of LH is supported by the fact that, conjugated forms of E_2 mimicked only the acute suppression of secretion of LH, without inducing the putative genomic actions of E_2 on secretion of LH or FSH. The presumed impermeability of conjugated forms of E_2 suggests that the plasma membrane is involved in mediating the acute effect of E_2. The ability of E_2-BSA and E_2-PEP to mimic only the acute action of E_2 justifies their further characterization as a tool for the study of acute, nongenomic plasma membrane-mediated actions of E_2.

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