

Blockade of Estrogen Action Upregulates Estrogen Receptor- α mRNA in the Fetal Brain

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Key Words

Sheep fetus · Hippocampus · Cerebellum · Faslodex · Fulvestrant

Abstract

Background: Fetal neuroendocrine maturation in late gestation is critical for maintenance of fetal homeostasis, growth, and readiness for birth. Sheep express estrogen receptors (ERs) in various brain regions. However, little is known about the regulation of ER- α and ER- β in the ovine brain prenatally. **Objective:** The present study was designed to test the hypothesis that the expression of ER is influenced by circulating estrogens in the late-gestation sheep fetus. **Methods:** Six chronically-catheterized twin fetal sheep were treated with vehicle or the ER blocker ICI 182,780 i.c.v. (0.25 μ g/day). Fetuses were sacrificed 6–14 days after surgery and start of infusion. Brain regions were rapidly isolated and snap-frozen for later extraction of mRNA and protein. ER- α and ER- β mRNA was measured using real-time PCR and protein was measured using Western blot. **Results:** Treatment with ICI 182,780 increased ER- α mRNA, especially in cerebellum and hippocampus. There were no changes in ER- α protein and no changes in ER- β at either the mRNA or protein level. **Conclusion:** Expression of ER- α is influenced by endogenous estrogens in the ovine fetal brain.

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Introduction

In the sheep, estrogen in fetal plasma largely originates in the placenta [1], creating an estrogen-rich environment in which the fetal brain matures [2]. It is known that the sheep expresses estrogen receptors (ERs) in various brain regions [3–5] and that ERs are expressed in the brains of other species prenatally [6]. The fetal brain and pituitary express both ER- α and ER- β , and the level of expression varies throughout the latter half of gestation when placental estrogen concentrations are changing [7]. We have recently reported that physiological increases in fetal plasma estradiol concentrations secondary to exogenous estradiol treatment increased the abundance of ER- α protein in brainstem, cerebellum, and cerebral cortex, and decreased ER- α protein in hypothalamus and ER- α mRNA in hippocampus [8]. Exogenous estradiol treatment did not alter the expression of ER- β in any brain region or in pituitary at either the protein or mRNA level [8]. Our previous studies with exogenous estradiol reveal the influence of increasing plasma estradiol concentrations, as occurs endogenously in the final stages of gestation. Throughout the latter half of gestation, however, the fetus develops in an estrogen-rich environment. What we do not know is whether the estrogens circulating in fetal blood exert a tonic influence on ER abundance. The present study was designed to test the hypothesis that

blockade of ERs, which would be expected to reduce estrogen action in tissues with ERs, alters the expression of ER regionally within the brain and pituitary in late-gestation fetal sheep.

Materials and Methods

Six time-dated pregnant ewes each carrying twin fetuses were prepared for study in vivo using chronic catheterization techniques [9]. Data from these experiments which are not reported in this paper have been reported elsewhere [10]. In each pregnancy, 1 fetus served as the experimental fetus while its twin served as an internal age-matched control. There were two experimental groups: ICI 182,780 i.c.v. (25 µg/day, n = 6; Tocris Biosciences, Bristol, UK) with the twin control (vehicle-infused) fetuses (n = 6). The dose of ICI 182,780 was calculated as approximately 20 times the mass of a known dose of exogenous estradiol known to stimulate fetal brain function, assuming that cerebral blood flow is approximately 5% of fetal combined ventricular output [11]. The ICI compound was dissolved in the vehicle, which was 50/50 water/dimethylsulfoxide. All fetuses were catheterized with femoral arterial, femoral venous, and amniotic fluid catheters, as well as a catheter into the lateral cerebral ventricle. For placement of the catheter into the lateral cerebral ventricle, the scalp was retracted and a small catheter (outside diameter 0.05 in, inside diameter 0.03 in) attached to an osmotic mini-pump (size 2mL4; Alza Corp., Palo Alto, Calif., USA) was inserted through a hole made in the skull. This catheter was held in place using VetBond (3M Corp., St. Paul, Minn., USA). The exposed catheter and osmotic mini-pump were placed subcutaneously before closing the incision on the head. The position of the intracerebroventricular catheter was confirmed at necropsy. Antibiotics (750 mg ampicillin) were administered into the amniotic cavity via direct injection. Finally, the hindlimb catheters were exteriorized through the flank of the ewe using a trochar, where they were maintained in a cloth pocket. Ewes were treated with 1 mg/kg flunixin meglumine (Webster Veterinary, Sterling, Mass., USA) for analgesia and returned to their pens where they were monitored until they could stand on their own. Twice daily during a 5-day recovery period, ewes were given antibiotic (ampicillin 750 mg i.m.) and rectal temperatures were monitored for indication of postoperative infection.

Following the recovery period, fetal blood samples were drawn from the arterial catheter every other morning (between 08:00 and 10:00 h) for use in hormone assays (data not reported). Blood gases were measured at the time of blood sampling using an ABL 77 Radiometer (Radiometer America, Inc., Cleveland, Ohio, USA) blood gas analyzer. On the 5th day postoperatively, the twin fetuses were euthanized (130–134 days' gestation at the time of sacrifice) with an overdose of sodium pentobarbital. Brains were rapidly removed, dissected into distinct regions, and snap-frozen in liquid nitrogen. The following tissues were collected as previously described: (1) brainstem, (2) hippocampus, (3) frontal cortex, (4) cerebellum, (5) hypothalamus, and (6) pituitary. Tissues were stored at -80°C until processed for mRNA or protein. Because of the small mass of tissue, the pituitaries were processed only for mRNA.

Tissues were individually pulverized using a Bio-Pulverizer (BioSpec Products, Bartlesville, Okla., USA), a trigger-style mor-

tar and pestle device. Total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions, and RNA concentration quantified as previously described [12]. After isolation, mRNA was stored in RNA Secure® (Ambion Corp., Austin, Tex., USA) or water at -80°C until use. RNA samples were converted into 4 µg stable cDNA by reverse transcription using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's instructions [12]. The resulting cDNA samples were stored at -20°C until real-time RT-PCR was performed.

ER- α , ER- β , and 18S rRNA gene expression was analyzed using real-time RT-PCR. ER- α and ER- β primer and probe sets were designed on Primer Express version 2.0 (Applied Biosystems) from known ovine sequences (accession Nos. AY059388 and NM_001009737, respectively) [13]. Sequences of probes and primers for ER- α and ER- β are reported in table 1. 18S ribosomal RNA was used as an internal control as previously described [14] and validated for developmental changes in gene expression in the developing brain [15]; primers and probe for 18S rRNA were purchased from Applied Biosystems (Cat. No. 4308329). ER- α and ER- β reactions contained 100 ng cDNA, 300 nM forward primer, 900 nM reverse primer, and 200 nM Taqman® probe. Probes for ER- α and ER- β were labeled with 6-carboxyfluoresceine (6-FAM) in the 5' position and carboxytetramethyl rhodamine (TAMRA) in the 3' position. Probe for 18S rRNA was labeled with VIC® in the 5' position and TAMRA in the 3' position. Control reactions run at the same time indicate the primers were specific for amplified product and that the RNA was not contaminated with genomic DNA. All reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reactions were amplified using the following conditions: 48°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fold changes in expression levels of the ER- α or - β genes within the brain regions of interest were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [16] using 18S rRNA as the internal reference.

For protein analysis, tissues were homogenized in boiling lysis buffer containing 1% SDS, 1 mM sodium orthovanadate, and 10 mM Tris pH 7.4 (Sigma Chemical Co., St. Louis, Mo., USA). Homogenates were boiled for 5 min then centrifuged at 7,500 g for 10 min at 4°C to remove particulate matter. The resulting supernatant was assayed for protein content using the BioRad DC Protein Assay (BioRad Laboratories, Hercules, Calif., USA) and stored at -80°C . Samples were diluted 1:1 with a denaturing loading buffer (4% SDS, 20% glycerol, 125 mM Tris pH 6.8, and 10% β -mercaptoethanol) and boiled for 5 min. Pre-cast 18-well 7.5% Criterion Tris-HCl gels (BioRad) were loaded with 40 µg protein per lane (25 µg for hippocampus) for SDS-PAGE. Gels were run for ~ 2.5 h at 100 V. Following electrophoresis, proteins were transferred onto nitrocellulose membrane at 22 V overnight, blocked for 1 h, and then incubated with antibodies specific for ER- α or ER- β for 1 h. ER- α antibody (MC-20 antibody, Cat. No. sc-542; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) was diluted 1:200 in antibody diluent (PBS with 0.05% Tween 20) and ER- β antibody (Cat. No. PA1-311, Affinity BioReagents, Golden, Colo., USA) was diluted 1:1,000 in antibody diluent (5% non-fat dry milk in PBS with 0.05% Tween 20, PBST). Membranes were visualized using goat peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co.; 1:5,000 for ER- α and 1:10,000 for ER- β) and ECL reagent (Amersham, Arlington Heights, Ill., USA), and developed on film. Quantity One densitometry software (BioRad)

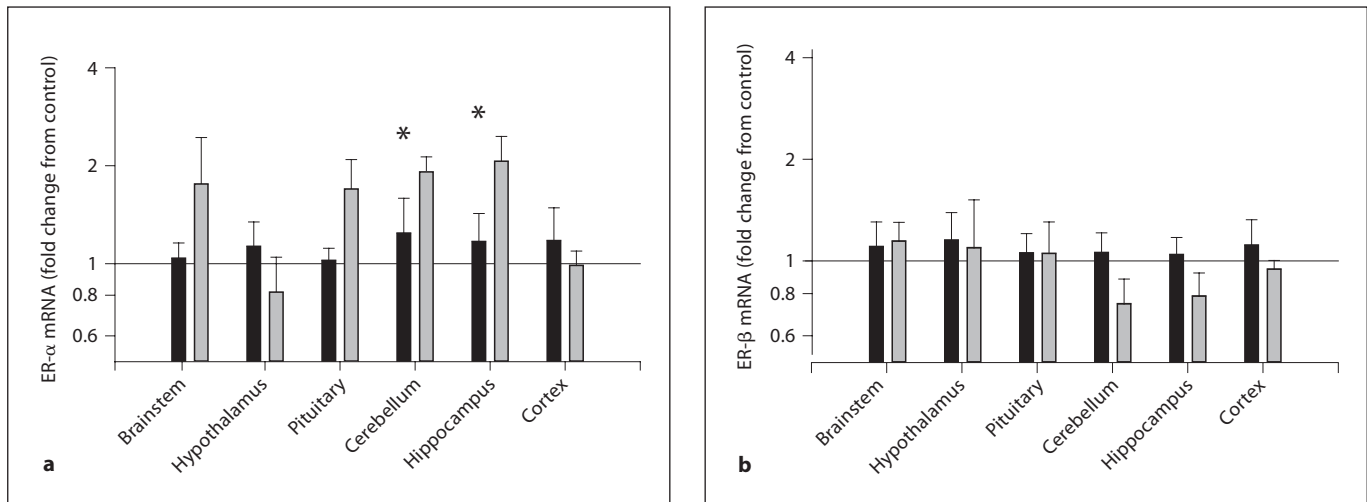


Fig. 1. ER- α (a) and ER- β (b) mRNA abundance in fetal brainstem, hypothalamus, pituitary, cerebellum, hippocampus, and cerebral cortex in untreated (black bars) and ICI-treated (grey bars) twin fetuses. mRNA abundances are expressed as fold changes from mean control values as calculated from the individual $\Delta\Delta\text{Ct}$ values and represented graphically in logarithmic bar graphs. Values are represented as mean \pm SEM. * Statistically significant differences between control and treated twins as tested by simple effects contrast analysis.

Table 1. ER primer and probe sequences used in real-time RT-PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	TaqMan probe
ER- α	AGGCACACGGGAGCACAT	TTCCATGGGCTTGTAGAAGTCA	CTTCCCTTCCTTCTCACTGTCTCAGCCC
ER- β	GCTCTGGTCTGGGTGATTGC	GTTAGCCAGGCGCATGGA	AAGAGCGGCATGTCCCTCCCAGCA

was utilized for blot analysis. Staining specificity was confirmed by preabsorption of the primary antibody using 25 μg ER- α synthetic peptide (Santa Cruz, Cat. No. sc-542 P) or 25 μg ER- β synthetic peptide (Affinity BioReagents, Cat. No. PA1-311) [7].

Statistics

Data were analyzed using two-way ANOVA, corrected for repeated measures in one dimension [17]. Comparisons of effect of ICI 182,780 within tissue were performed using simple effects contrast analysis after ANOVA [18]. All statistical calculations were performed using SPSS Version 15 (SPSS Corp., Chicago, Ill., USA). The criterion used for assessing statistical significance was $p < 0.05$.

Results

Blockade of ERs with ICI 182,780 altered the gene expression of ER- α , but did not alter the expression of the ER- β gene or the abundance of either ER- α or ER- β at the

protein level. Intracerebroventricular infusion of ICI 182,786 at a rate of 25 $\mu\text{g}/\text{day}$ resulted in an overall statistically significant increase in ER- α abundance at the mRNA level (fig. 1; $p < 0.05$ by two-way ANOVA) but not at the protein level (table 2). The increase in ER- α expression was statistically significant in the hippocampus and cerebellum at the mRNA level (as demonstrated using simple effects contrast analysis; fig. 1). ER- β was not altered significantly at either the mRNA (fig. 1) or protein levels (table 1).

Infusion of ICI 182,780 did not alter fetal blood gases. There were no differences in arterial oxygen tension (18.2 ± 0.9 and 17.7 ± 1.2 mm Hg, in vehicle- and ICI-treated fetuses, respectively), arterial carbon dioxide tension (56.4 ± 0.8 and 56.9 ± 0.9 mm Hg in vehicle- and ICI-treated fetuses, respectively) or pH (7.34 ± 0.01 and 7.35 ± 0.01) between groups. These blood gas values have been reported elsewhere [10].

Table 2. ER- α and ER- β protein responses to ICI 182,780¹

Tissue	ER- α		ER- β	
	control twin	ICI twin	control twin	ICI twin
Pituitary			N/A	N/A
Hypothalamus	1,165 \pm 124	1,064 \pm 56	396 \pm 65	424 \pm 82
Cerebellum	600 \pm 74	580 \pm 113	897 \pm 110	898 \pm 104
Brainstem	624 \pm 35	650 \pm 51	744 \pm 100	801 \pm 105
Cortex	996 \pm 88	1,047 \pm 67	932 \pm 53	1,063 \pm 77
Hippocampus	1,657 \pm 54	1,760 \pm 110	1,261 \pm 90	1,360 \pm 97

¹ Data are represented as mean \pm SEM of values of optical density (arbitrary units).

Discussion

We believe that the present study is the first to investigate the influence of endogenous estrogen on ER mRNA abundance in the brain and pituitary of the late-gestation fetus. This experiment demonstrates that ER- α gene expression in ovine fetal brain and pituitary is sensitive to changes in estrogen availability. These results suggest that in the late-gestation fetal sheep, concentrations of estrogens derived from endogenous sources are sufficient to modify ER- α gene expression by receptor autoregulation [19, 20]. The effect of the ER blocker ICI 182,780 up-regulating the mRNA expression of the ER- α gene was statistically significant when including all of the data in the analysis, but was particularly evident in cerebellum and hippocampus (fig. 1). Interestingly, ER- α is generally responsive to changes in estrogen while ER- β is not. It is also possible, perhaps likely, that there are changes in receptor turnover that alter the relative changes in protein relative to that of mRNA for each receptor [21].

The increase in ER- α mRNA was not mirrored by similar changes at the protein level. We suspect that this might have resulted from a change in receptor turnover rate, and that the increased mRNA was a sign of an increased net synthesis rate. This may have been caused by an increase in receptor degradation in response to the binding of ligand to the receptor [21, 22]. On the other hand, the ER at the mRNA level is known to be down-regulated by estrogen [23], and in mammary gland this is associated with decreases in STAT5 phosphorylation [24]. Yet another possibility is that ICI 182,780 treatment resulted in a change in ER- α mRNA translation efficiency. Kos et al. [25] have demonstrated that there are multiple promoters in the ER- α gene, effectively allowing for multiple polyadenylation states that introduce differences in the efficiency of translation of the ER- α mRNA. We

therefore interpret our results as being consistent with an increase in receptor transcription, an increase in receptor protein degradation, a decrease in receptor mRNA translation efficiency, or some combination of all three possible cellular responses to ICI 178,182.

The results of the present study complement the results of a recent study from this laboratory demonstrating that increases in fetal plasma estradiol influence ER- α expression in brainstem, hypothalamus, cerebellum, and cerebral cortex in late-gestation fetal sheep [8]. Interestingly, the exogenous estradiol increased ER- α mRNA in hippocampus and ER- α protein in brainstem, cerebellum, and cerebral cortex, and decreased ER- α protein in the hypothalamus. In that study, the mismatch between changes in mRNA and protein suggested to us that increases in fetal plasma estradiol concentration might have changed the rate of turnover of the receptor protein [8]. It is not clear that the effects of ICI 182,780 in the present study or the effects of estradiol in the previous study are the result of direct influence on ER- α transcription. While the present study does not reveal the mechanism of the change in ER- α gene expression, the study does demonstrate that the expression of the ER- α gene is in part a result of the circulating concentrations of ligand.

We have proposed [2] that there is a positive feedback cycle involving the placental production of estrogen, which is increased at the end of gestation by cortisol in the sheep [26], and the activity of the fetal hypothalamus-pituitary-adrenal axis, which is stimulated by physiological increases in fetal plasma estradiol concentrations [27]. The results of this and other studies from this laboratory demonstrate that several physiological variables interact to alter the gene expression and receptor abundance of both ER- α and ER- β in fetal brain regions important for control of the fetal HPA axis. We have reported that ER- α and ER- β expression are dramatically up-

regulated during the response to cerebral hypoperfusion, suggesting that activation of specific reflex pathways within the brain might increase ER abundance in ER-expressing neurons within that pathway [8]. The changes, therefore, in the abundance of receptor protein throughout the latter half of gestation [7], might indicate dynamic changes in estrogen responsiveness of the neuroendocrine elements that ultimately control stress responsiveness and timing of parturition in this species [2].

In summary, the results of the present study support the conclusion that withdrawal of estrogen increases ER- α

gene expression in the brain and pituitary of the late-gestation fetus. It is possible, therefore, that ontogenetic changes in ER- α expression are partly the result of changes in estrogen biosynthesis and bioavailability in the fetus.

Acknowledgements

We thank Ms. Xiaoying (Lisa) Fang for her technical expertise and Maureen Keller-Wood, PhD, for her advice and consultation. This work was supported by HD42135 from NICHD to C.E.W.

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