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Neonatal Exposure to Estrogens Suppresses Activin Expression and Signaling in the Mouse Ovary

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In the ovary, the steroid hormone estrogen and the TGF-β superfamily member activin are both produced by granulosa cells and they both have intraovarian functions. Emerging evidence has indicated an interaction of these two signaling pathways. Based on the fact that estrogen and activin can impact early follicle formation and development, we hypothesize that estrogen treatment may alter activin signaling in the neonatal ovary. Therefore, this study was designed to examine the effect of neonatal diethylstilbestrol (DES) and estradiol (E_2) exposure on the mRNA and protein levels of the key factors involved in activin signaling in the mouse ovary. CD-1 mouse pups were given daily injections of DES, E_2, or oil on postnatal d 1–5, and ovaries and sera were collected on d 19. Neonatal DES or E_2 exposure decreased the number of small antral follicles, induced multioocytic follicle formation, and decreased activin β-subunit mRNA and protein levels. Consistent with local loss of β-subunit expression, the phosphorylation of Smad 2, a marker of activin-dependent signaling, was decreased in the estrogen-treated ovaries. The decreased β-subunit expression resulted in a decrease in serum inhibin levels, with a corresponding increase in FSH. Estrogen also suppressed activin subunit gene promoter activities, suggesting a direct transcriptional effect. Overall, this study demonstrates that activin subunits are targets of estrogen action in the early mouse ovary. (*Endocrinology* 148: 1968–1976, 2007)

OVARIAN FOLLICLE DEVELOPMENT is a complicated process finely regulated by various intrinsic and endocrine factors, and this process involves interactions between multiple cell types within the ovary. Factors produced by ovarian granulosa cells include the steroid hormone estrogen and the TGF-β superfamily member activin, both of which have been demonstrated to play an important intraovarian role in regulating follicle development (1–9).

Estrogen signals through binding to estrogen receptors, ERα or ERβ, in most cases, although receptor-independent mechanisms exist. Both ERα and ERβ are expressed in mouse or rat ovaries, where ERβ is the most abundant form of ER and is expressed predominantly in the granulosa cells, whereas ERα is expressed mostly in the theca cells (10, 11). The importance of estrogen in ovarian follicle development and maturation has been elucidated in ERα-, ERβ-, or ERα- and ERβ-compound knockout mouse models, all of which show various defects in ovulation and/or follicle development (12–15). ERα-knockout mice are infertile, and ovarian follicles fail to mature or ovulate and form hemorrhagic cysts (12, 13). ERβ-knockout mice are either subfertile or infertile, and ovarian follicles are relatively normal, although the numbers of large antral follicles and corpora lutea are reduced (12, 14, 15). ERα- and ERβ-compound knockout mice are also infertile and have a similar phenotype to that of ERα-knockout mice. In addition, antral follicles in ERα- and ERβ-compound knockout mice are smaller in size and contain fewer granulosa cells (12). Unexpectedly, Sertoli cells are also found in the ERα- and ERβ-compound knockout mouse ovaries, suggesting an important role for ERs in maintaining granulosa cell fate (12).

An impact of estrogen on the proper formation and long-term function of the early follicle pool has been indicted by many studies. In an estrogen-deficient mouse model, the aromatase knockout mouse, there is a blockage of follicle development at the antral stage and absence of corpora lutea, as well as a decrease in primordial and primary follicle numbers (16, 17). When administered neonatally during the critical period of follicle formation in rodents, estrogens can induce several long-term pathologies in the ovary. For instance, delayed follicle and interstitial development at d 14 and 21 of age in neonatal estradiol benzoate exposed rats have been documented (18). A lack of corpora lutea in adult mice exposed neonatally to diethylstilbestrol (DES) or estradiol (E_2) has also been reported (19), suggesting that these effects persist and impact fertility. Neonatal exposure to DES, E_2, or the phytoestrogen genistein in mice also induces formation of multioocytic follicles (MOFs) (20–22), which have also been reported in alligators exposed to environmental estrogenic contaminants (23).

Activin and its functional antagonist inhibin were originally isolated from gonadal sources based on their ability to stimulate (activin) or suppress (inhibin) the synthesis and secretion of FSH (24–30). Activin and inhibin are structurally related and share a common signaling pathway. Activin is a dimer of two β-subunits, βA or βB, to form activin A (βAβA), activin B (βBβB), or activin AB (βAβB), and inhibin is a heterodimer of a unique α-subunit with either of the two shared β-subunits to form inhibin A (αβA) or inhibin B (αβB)

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Abbreviations: CV, Coefficient of variation; DES, diethylstilbestrol; E_2, estradiol; MOF, multioocytic follicle; P-Smad 2, phosphorylated-Smad 2.

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(26–29). Activin signals through a receptor serine-threonine kinase/Smad protein pathway, which involves activin binding receptors (ACTR IIA and ACTR IIB), a signaling receptor (ACTR IB), signaling coactivators (Smads 2, 3, and 4), and an inhibitor (Smad 7) (31–34). Inhibin antagonizes activin action by competing for the β-subunits or for binding to activin type II receptors (35). A coreceptor, β-glycan, mediates the latter action (36, 37). Activin has been shown to regulate ovarian granulosa cell proliferation and differentiation (3, 5, 6, 38), promote ovarian follicle atresia (39), increase FSH receptor expression in undifferentiated granulosa cells (40, 41), and stimulate oocyte maturation *in vitro* (42). Inhibin is able to stimulate LH-dependent androgen production by thecal cells (43, 44). Mice that lack activin receptor type II are infertile, and follicle development is blocked at the antral stage with very few corpora lutea observed (45). Overexpression of follistatin, an activin antagonist, in transgenic mice also blocks follicle development at the secondary follicle stage (46).

Our laboratories have developed two transgenic mouse models with defects in activin expression or activin signaling (47, 48). These are mice that overexpress the inhibin α-subunit gene from a metallothionein-I promoter (MT-α inhibin subunit), which have decreased activin levels (47), and mice that express a nonphosphorylatable Smad 2 protein from the Müllerian inhibiting substance promoter (MIS-Smad 2 dominant negative), which have interrupted activin signaling (48). In these mice, a variety of ovarian pathologies have been observed, including development of ovarian cysts and formation of MOFs (47, 48). Because formation of MOFs likely occurs during the earliest stages of follicle formation, probably through an incomplete breakdown of germ-cell syncytia (49), these transgenic mouse models indicate an important role for activin in the early ovary and in establishing the follicle pool. This is re-enforced by studies showing that administration of excess activin in the neonatal period enhances the number of primordial follicles (50).

Estrogen and activin signaling proteins are colocalized in the granulosa cells in the ovary, suggesting a possible interaction of these two factors. Indeed, multiple findings have indicated an effect of estrogen on activin signaling pathways (51–57). Based on the fact that estrogen and activin both have an impact on early ovarian follicle formation and follicle development, we hypothesize that estrogen treatment may alter activin signaling in the neonatal ovary. Therefore, this study was designed to examine the effect of neonatal DES and E_2 exposure on the mRNA and protein levels of the key factors involved in activin signaling in the mouse ovary. The results demonstrate that neonatal estrogen exposure decreases activin subunit gene expression and impacts activin signaling, indicating that activin genes are targets of estrogen action in the mouse ovary.

Materials and Methods

Antibodies

Rabbit polyclonal antibodies against inhibin α, βA, and βB subunits were gifts from W. Vale and J. Vaughn (The Salk Institute, La Jolla, CA). Rabbit polyclonal antibody against actin was purchased from Sigma (St. Louis, MO). Rabbit anti-Smad2 and Phospho-Smad2 antibodies were purchased from Invitrogen (Carlsbad, CA) and Cell Signaling (Danvers, MA), respectively. HRP-labeled donkey antirabbit IgG was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Biotin-labeled goat antirabbit IgG was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Animal treatment and tissue collection

CD-1 mice (Harlan, Indianapolis, IN) were maintained on a 12-h light/12-h dark cycle (lights off at 1700 h) with food and water available *ad libitum*. Breeders (90–180 d old) were fed with a soy-free mouse chow (Harlan 7926) to limit exogenous phytoestrogen intake through food. At the time of delivery (d 1), eight pups were kept with each female to minimize the possible difference in pup development caused by nutrient availability. The pups were given daily sc injections of 1 μg DES (0.5 mg/kg), 20 μg E_2 (10 mg/kg) or corn oil (vehicle), all in 20 μl of volume, on d 1–5 after birth. Ovaries and sera were collected on postnatal d 19. The injection doses were chosen based on previous reports (20–22). Ovaries were collected on d 19 because, at this age, all follicles through the antral stage can be observed and yet the animals are prepubertal. Ovaries were either stored at −80 C for later RNA isolation or ovary protein extract preparation, or immediately fixed for follicle counting and IHC studies. Animals were cared for in accordance with all federal and institutional guidelines.

RNA isolation and real-time PCR

Total RNA was isolated from d 19 ovaries using a Qiagen RNA isolation kit (Qiagen, Valencia, CA). On column DNase digestion was performed using an RNase-Free DNase Set (Qiagen) to eliminate DNA contamination, and the resulting RNA was reverse transcribed with AMV-reverse transcriptase (Fisher Scientific, Pittsburgh, PA). Real-time PCR was performed on a Bio-Rad iCycler using SyberGreen SuperMix (Bio-Rad Laboratories, Inc., Hercules, CA) to quantitatively measure the mRNA levels of inhibin α, activin βA, activin βB, ACTR IA, ACTR IIA, ACTR IB, ACTR IIB, Smad 2, Smad 3, Smad 4, Smad 7, and follistatin. Primers were designed according to the complete mouse cDNA sequences of the above genes. A list of the primers used is shown in Table 1. Ribosomal protein L19 was used as an internal control for all reactions. The threshold cycle numbers of L19 were not altered by neonatal DES or E_2 treatment compared with the oil controls (data not shown). The amplicons from reactions for the activin/inhibin subunits were sequenced to confirm correct products. Specificity of all the real-time PCR

TABLE 1. List of primers used in this study

Genes	PCR primers (5′–3′)	Product size (bp)
L19	CTG AAG GTC AAA GGG AAT GTG	195
	GGA CAG AGT CTT GAT GAT CTC	
Inhibin α	CTC CCA GGC TAT CCT TTT CC	112
	TGG CCG GAA TAC ATA AGT GA	
Inhibin βA	GAT CAT CAC CTT TGC CGA GT	143
	TGG TCC TGG TTC TGT TAG CC	
Inhibin βB	CTA GAG TGT GAT GGG CGG AC	308
	ACA TCC CGC TTG ACA ATG TT	
ACTR IA	TGT ACT GTC CAT AGC CAG CG	131
	TGC AGC ACT GTC CAT TCT TC	
ACTR IB	CGC TCC AGG ATC TCG TCT AC	202
	AAC CAA GAC CGT TCT TCA CG	
ACTR IIA	AGT GAA GCA AGG TTG TTG GC	128
	CAT ATT GCC CTC ACA GCA AC	
ACTR IIB	TCA ATT GCT ACG ACA GGC AG	140
	TGG CTC GTA CGT GAC TTC TG	
Smad 2	CTC CAG TCT TAG TGC CTC GG	300
	AAC ACC AGA ATG CAG GTT CC	
Smad 3	GAC CAC AGA AGA AAA CCC CA	245
	AGG GCA TGC TCC ATT ACA AC	
Smad 4	CCT GTT GTG ACT GTG GAT GG	159
	CCA AAC GTC ACC TTC ACC TT	
Smad 7	CAG CTC AAT TCG GAC AAC AA	193
	AAC CAG GGA ACA CTT TGT GC	
Follistatin	GAG TGA CTT ACT CCA GCG CC	266
	AGC TTC CTT CAT GGC ACA CT	

was also confirmed by a single peak in the melt curves and by a single band of the predicted size after agarose gel electrophoresis of the PCR products (data not shown).

Western blot

Protein homogenates were prepared in GBA buffer [50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10% glycerol, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, Roche Molecular Biochemicals, Indianapolis, IN), and 0.1 mM bacitracin (Sigma), pH 7.4 at 4 C] from pools of ovaries collected from four to six mice. Proteins were electrophoresed under reducing conditions in 13% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were incubated overnight at 4 C with primary antibody (anti-inhibin α, βA, βB, or anti-Phospho-Smad2, 1:1000 dilution; anti-Smad2, 1:500 dilution) followed by 1 h of incubation at room temperature with HRP-labeled donkey antirabbit secondary antibody (1:5000 dilution). Proteins were then visualized by chemiluminescence. For inhibin α, βA, and βB detection, the experiments were repeated three to four times and the blots were scanned by densitometry. The intensities of the protein bands were analyzed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). The pixel intensity of each precursor or mature protein band was normalized against that of the corresponding loading control, which was actin. The relative intensity of the precursor or mature protein band was then obtained from the ratio of the experimental group (DES or E_2) over the oil control.

Immunohistochemistry

Fresh ovaries obtained from mice from each treatment group were fixed in 4% PFA overnight and embedded in paraffin. Five-micrometer serial sections were obtained and mounted on Superfrost-Plus slides (Fisher Scientific). Sections from each ovary were either left unstained for use in immunohistochemistry or stained with hematoxylin and eosin to examine the numbers of follicles. For immunohistochemistry, slides were deparaffinized and rehydrated. Antigen retrieval was performed using 0.01 M sodium citrate. Each tissue section on the slides was treated with 3% H_2O_2 and avidin/biotin blocking reagents (Vector Laboratories, Inc.). Tissue sections were then incubated with primary antibody [anti-Phospho-Smad2 (1:1000 dilution) or anti-Smad2 (1:250 dilution)] overnight at 4 C and incubated the next day with secondary antibody (biotinylated goat antirabbit IgG, 1:200 dilution) at room temperature for 30 min. Sections were then treated with ABC reagent (Vectastain Elite ABC kits; Vector Laboratories, Inc.). For visualization, a TSA Plus Fluorescein System (PerkinElmer, Boston, MA) was used according to the manufacturer's instruction. Sections were counterstained with DAPI (data not shown). Negative controls were produced by omitting the primary antibody. No fluorescent signal was detected in the negative controls, indicating specificity of the assay (data not shown).

GRMO2 cell culture and transfection and luciferase assays

GRMO2 cells are from a mouse granulosa cell line provided by N.V. Innogenetics (Ghent, Belgium). Culture of GRMO2 cells and transfection and luciferase assays were performed as described previously (60) with slight modification. Briefly, cells were cultured in a humidified incubator at 37 C and 5% CO_2 in a phenol red-free D-MEM/F-12 medium (Invitrogen Corporation, Grand Island, NY) supplemented with 10 μg/ml insulin, 5 nM sodium selenite, 5 μg/ml transferring, 100 μg/ml sodium pyruvate, and 2% charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT). After 3-d culturing in the aforementioned estrogen-deprived condition, GRMO2 cells were transiently transfected with DNA constructs (500 ng per well of a 12-well culture plate) using cationic liposomes in a phenol red-free Opti-MEM (Invitrogen Corporation). The DNA constructs used were either a 837-bp (−769 to +68) inhibin α-subunit promoter-luciferase reporter construct (61), a 609-bp (−571 to +38) βA subunit promoter-luciferase reporter construct (62), or a 547-bp (−1460 to −914) βB subunit promoter-luciferase reporter construct (63). After 6 h transfection, cells were aspirated and maintained in fresh culture medium for 14–16 h. Fresh medium containing vehicle, 100 nM E_2, 1 μM ICI182, 780, or a combination of the latter two was then given to the cells for 24 h. After the treatments, cells were washed with PBS and lysed on ice in lysis buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100] for 20 min. For luciferase assay, cell lysates (100 μl) were added to 400 μl of reaction buffer (25 mM HEPES, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 2.5 mM ATP, 1 mM dithiothreitol, 1 μg/ml BSA) and 100 μl of 1 mM luciferin (sodium salt) (Analytical Bioluminescence, San Diego, CA) were added using an automatic injector and emitted luminescence was measured using a 2010 luminometer (Analytical Bioluminescence) for 10 sec. Relative light units were normalized for total protein content measured with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.).

Hormone measurements

Total activin A (free activin A plus follistatin-bound activin A, dimeric form) in serum and in ovary was measured using a total activin A ELISA kit (Oxford Bio-Innovation LTD, Oxfordshire, UK) according to the manufacturer's instructions. Ovary extracts were prepared as described previously (64, 65) with slight modification. Briefly, three to four d-19 ovaries from each treatment group were homogenized in 150 μl of 0.85% (wt/vol) NaCl using a sonic disruptor (Model 100; Fisher Scientific). Homogenates were then centrifuged at 15,000 × *g* for 30 min at 4 C. The supernatants were collected and immediately used for total activin A ELISA. Intraassay and interassay coefficients of variation (CV) were less than 8% and assay sensitivity was 78 pg/ml. The results were normalized with protein concentrations of the ovary extracts measured by Bradford assay. Serum inhibin A (CV < 6%; sensitivity, 20 pg/ml), inhibin B (CV < 10%; sensitivity, 20 pg/ml), FSH (CV < 7%; sensitivity, 3.2 ng/ml), and LH (CV < 9%; sensitivity, 0.08 ng/ml) levels from each treatment group were measured by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

Follicle counting

Follicle counting was performed as reported previously (66–68). Primordial follicles included those that had an oocyte surrounded by a single layer of squamous granulosa cells. Primary follicles had an enlarged oocyte surrounded by either a single layer of cuboidal granulosa cells or a single layer of mixed cuboidal and squamous granulosa cells. Secondary follicles were follicles that had an enlarged oocyte surrounded by two or more layers of cuboidal granulosa cells but had no fluid-filled antrum. Small antral follicles had an oocyte surrounded by layers of cuboidal granulosa cells that contained one or more small antra. Large antral follicles had an oocyte enclosed by cumulus cells and a single large antrum surrounded by a single layer of cuboidal granulosa cells. Atretic follicles were those that had a degenerating or fractionated oocyte. Follicles with oocytes containing a clear nucleus were counted on every fifth section of each serial sectioned ovary. The cumulative counts for each ovary were then multiplied by a correction factor of five to estimate the total number of follicles (66–68). Because formation of MOFs was observed in these neonatal estrogen-treated mice, the numbers of MOFs were also counted on every fifth section of each serial sectioned ovary. Each counted MOF was compared with those on the previous and following counted sections to avoid double counting. The cumulative counts for each ovary were then multiplied by a correction factor of five.

Statistics

Data are presented as means ± SEM. One-way ANOVA followed by a Tukey-Kramer *post hoc* analysis was used for statistical comparisons among multiple groups. For statistical comparisons between two groups, the Student's two-tailed *t* test was used. $P < 0.05$ was considered significant.

Results

Effect of neonatal DES or E_2 treatment on activin subunit gene expression and protein levels

To determine whether neonatal estrogen treatment altered activin expression or signaling in the ovary, we first sought to examine gene expression levels of activin/inhibin subunits and activin signaling components. We injected CD-1 mouse pups with oil (vehicle control), DES, or E_2 on d 1–5

after birth, collected ovaries on d 19, and isolated total RNA. Ovaries were collected on d 19 because, at this age, all follicles through the antral stage can be observed and yet the animals are prepubertal. The mRNA abundance of activin/inhibin subunits and activin signaling proteins was then measured by quantitative real-time PCR in the oil and estrogen-treated groups. A 75% decrease in the βA subunit and 60% decrease in the βB subunit mRNA levels were observed in the neonatal DES-treated mouse ovaries (Fig. 1A). In the neonatal E_2-treated mouse ovaries, the mRNA levels of βA and βB subunits decreased by about 80 and 75%, respectively (Fig. 1B). The mRNA levels of inhibin α also decreased about 25% in both groups, although this was significant only in the E_2-treated mice. The mRNA abundance of activin binding receptors (ACTR IIA and ACTR IIB), signaling receptor (ACTR IB), signaling coactivators (Smads 2, 3, and 4), and inhibitor (Smad 7) as well as follistatin was not significantly changed after the two estrogen treatments (data not shown).

Because significant decreases in the mRNA levels of the activin βA and βB subunits were observed in the neonatal DES- or E_2-treated mouse ovaries, we examined the protein abundance of the inhibin α, and βA and βB subunits. In whole ovary homogenates from all treatment groups, both precursor and mature forms of the three inhibin/active subunit proteins were detected by Western blots (Fig. 2, A and B). Quantitative results of multiple Western blots for each subunit are shown in Fig. 2, C and D. In DES-treated mice, βA subunit precursor and mature protein levels were 75 and 62%, respectively, of those from the controls (Fig. 2C). In E_2-treated mice, βA subunit precursor and mature protein levels were 69 and 73%, respectively, of those from the controls (Fig. 2D). Inhibin α precursor and mature protein levels were not significantly altered by either of the estrogen treatments. Mature protein levels of βB subunit were slightly decreased in the ovaries from DES or E2-treated mice, although the decreases were not statistically significant. The minor differences between mRNA level measurements and protein level measurements may be explained by different sensitivities between quantitative real-time PCR and Western blot, or could reflect differences in processing or stability of the βB subunit compared with βA.

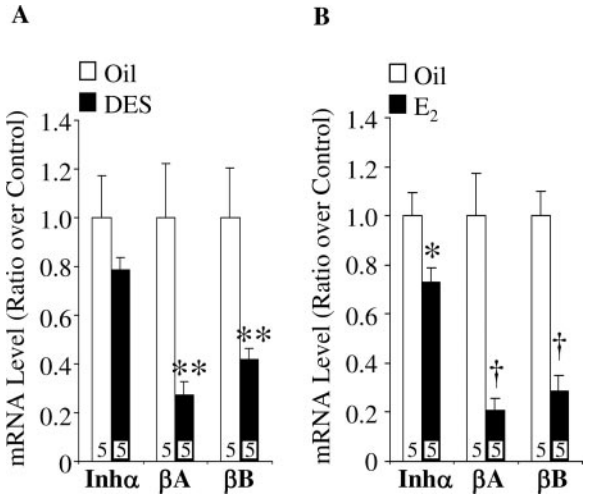

FIG. 1. Real-time quantitative PCR analysis of inhibin/activin subunit mRNA levels in mouse ovaries after neonatal DES or E_2 treatment. Whole ovaries from each treatment group were used for RNA isolation followed by RT and real-time PCR. A, Measurements in ovaries from neonatal DES-treated animals. B, Measurements in ovaries from neonatal E_2-treated animals. Significant differences between oil and estrogen-treated groups are indicated above the bars. The *numbers at the bottom of each bar* indicate experimental replicates. *, $P < 0.05$; **, $P < 0.005$; †, $P < 0.001$.

To further confirm the mRNA and Western blot results and also to measure the biologically active dimeric form of activin in the whole ovary, ELISA were performed. Because an ELISA for total activin is only available for activin A, we measured this isoform in whole ovary extracts. The results show that total activin A levels decreased in the ovaries of mice treated with estrogens, compared with the oil controls (Table 2). There was no significant difference between the DES- and E_2-treated groups.

Effect of E_2 on the inhibin α, βA, and βB subunit promoter activities

To investigate the mechanism of the estrogen effect on activin gene expression and to determine whether this represents a direct transcriptional effect, we examined inhibin α, βA, and βB subunit promoter activities in transfected GRMO2 granulosa cells treated with vehicle (control), E_2, ICI182, 780, or a combination of the latter two. GRMO2 cells are a cell line derived from undifferentiated mouse granulosa cells collected from follicles at early developmental stages that express the endogenous inhibin and activin subunit genes. Results showed that, although E_2 did not alter inhibin α promoter activity (Fig. 3A), it had a relatively small but significant suppressive effect on the βA and βB subunit promoter activities (Fig. 3, B and C, respectively), consistent with its effect on β-subunit mRNA levels. Most importantly, the suppressive effect of E_2 on the βA and βB subunit promoter activities was abolished by the anti-estrogen ICI182, 780, indicating a specific estrogenic effect (Fig. 3, B and C). Interestingly, ICI182, 780 decreased inhibin α promoter activity when given alone or in combination with E_2 (Fig. 3A). This decrease in inhibin α promoter activity may result from a suppression of endogenous estrogen in the culture medium by the excess amount of ICI182, 780.

Immunohistochemistry and Western blot of total or phosphorylated-Smad 2 (P-Smad 2) in the neonatal DES- or E_2-treated mouse ovaries

Because activin mRNA and activin A protein levels were decreased in the ovaries from mice treated neonatally with DES or E_2, we examined activin signaling status in those ovaries. Activin signaling involves phosphorylation of Smad proteins, including Smad 2 and Smad 3. Therefore, immunohistochemical studies and Western blot analysis of P-Smad 2 and total Smad 2 were performed. Consistent with decreased activin expression, a decrease in P-Smad 2 levels was observed at the whole ovary level in the neonatal DES- or E_2-treated mouse ovaries compared with the oil controls, whereas total Smad 2 levels were not different among the groups (Fig. 4A, ×100 pictures). In all ovaries, P-Smad 2 was detected predominantly in nuclei and Smad 2 was detected mostly in the cytoplasm (Fig. 4A, ×200 pictures). The de-

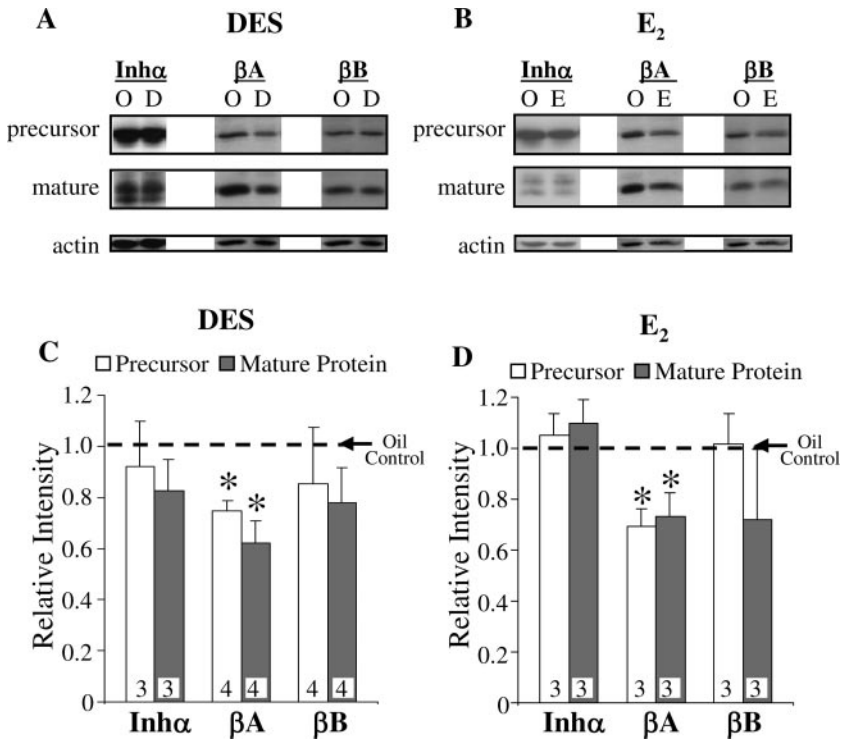

FIG. 2. Western blot measurement of inhibin α, βA, and βB subunit protein levels. A, Representative pictures of Western blots. Both precursor and mature forms of all three subunits were detected in all treatment groups. Actin was used as a loading control. O, Oil; D, DES. B, Quantitative results of Western blots from the neonatal DES-treated mouse ovaries as compared to the oil controls. E, E_2. C, Quantitative results of Western blots from the neonatal E_2-treated mouse ovaries as compared to the oil controls. The *numbers at the bottom of each bar* indicate experimental replicates. *, $P < 0.05$.

crease in P-Smad 2 levels was most apparent in the granulosa cells, and was not observed in germ cells (Fig. 4A, ×200 pictures). Similar results were also obtained for P-Smad 3 and total Smad 3 (data not shown). Western blot confirmed the decrease in P-Smad 2 protein levels in the ovaries after DES or E_2 treatment as compared to the oil controls (Fig. 4B).

Hormone measurements in the neonatal DES- or E_2-treated mice

Because activin and inhibin share common β-subunits and we observed a decrease in β-subunit expression in neonatal DES- or E_2-treated mouse ovaries, we measured serum total activin A, inhibin A, and inhibin B concentrations in 19-d-old mice treated neonatally with oil, DES, or E_2. In addition, because activin and inhibin regulate the synthesis and secretion of FSH, we also measured serum FSH and LH concentrations. Serum total activin A levels were 694.3 ± 25.5 pg/ml in oil-treated (n = 9), 640.3 ± 62.3 pg/ml in DES-treated (n = 4), and 545.5 ± 56.4 pg/ml in E_2-treated (n = 6) mice. The differences among these three groups were not significant as tested by ANOVA. Because we did observe a decrease in ovarian activin levels (Table 3), this suggests that ovarian activin may not all be secreted and the ovary may not be the only/major source of circulating activin (69, 70). However, serum inhibin A and inhibin B concentrations in the DES- or E_2-treated animals were both lower than those in the oil controls (Fig. 5, A and B, respectively). This observation is consistent with decreased β-subunits in the ovary, because inhibin and activin share the same β-subunits and the ovary is considered the major source of circulating inhibin (70, 71). FSH concentrations increased in the DES- or E_2-treated mice compared with the oil controls, and the increase in the DES-treated mice was more robust than that in the E_2-treated mice (Fig. 5C). The elevation in FSH levels in the DES or E_2-treated mice is likely a consequence of decreased inhibin levels, rather than a consequence of any change in GnRH, as serum LH levels were not altered by any of the treatments (Fig. 5D). To examine whether the elevation in FSH levels is a chronicle phenomenon after neonatal estrogen exposure, serum FSH levels were also measured in 6-d-old mice treated neonatally with oil, DES, or E_2. The results revealed that, unlike what was observed in 19-d-old animals, at d 6, serum FSH levels were actually decreased in the DES- or E_2-treated mice (7.9 ± 0.3 and 7.9 ± 0.9 ng/ml, respectively) compared with the controls (24.6 ± 1.5 ng/ml). This observation is consistent with reports by others (72).

TABLE 2. ELISA measurement of total activin A levels in the ovary

Treatment	Total activin A (pg/mg protein)
Oil	220.8 ± 22.4[a]
DES	126.1 ± 28.1[b]
E_2	78.7 ± 11.6[b]

Data are presented as mean ± SEM. n = 3–5 animals. *Different superscripts* indicate statistical significance at $P < 0.01$.

Changes in ovarian weight and follicle populations after neonatal DES or E_2 treatment

To evaluate the impact of neonatal estrogen treatment on ovarian follicle development and thus to see whether this might contribute to decreased activin expression in the ovary, we collected ovaries on d 19 from neonatal oil, DES-treated, or E_2-treated mice, measured ovarian weight, and counted the number of follicles. The ovarian weight was lower in DES- or E_2-treated animals than in oil-treated ani-

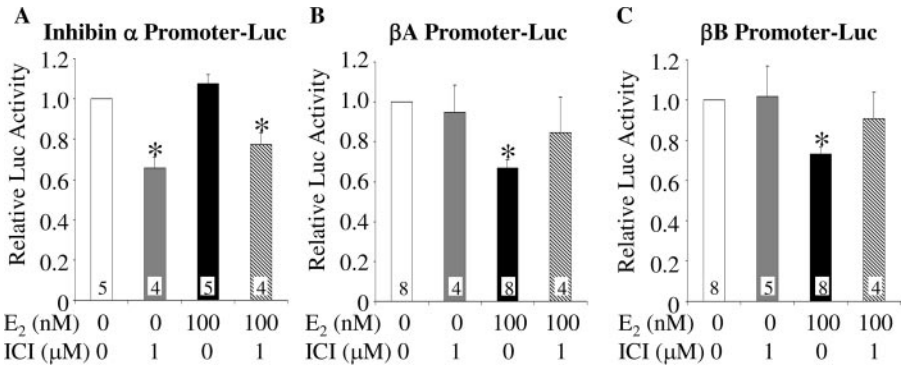

FIG. 3. Effects of E_2 and ICI182, 780 on inhibin α-, βA-, and βB-promoter activities in transfected GRMO2 granulosa cells. All the treatments were given for 24 h. The *numbers at the bottom of each bar* indicate replicate experiments. Each measurement was done in triplicate. *, $P < 0.05$.

mals (Table 3). In addition, DES treatment resulted in fewer total and small-antral follicles. E_2 also decreased the number of small-antral follicles. The number of large-antral follicles appeared to be reduced in DES or E_2-treated animals, although this was not statistically significant given the small number of these advanced follicles at this age (Table 3). Thus, neonatal estrogen treatment had a marked effect on the ovarian follicle pool, and this may contribute to the decreased gene expression and biosynthesis of activin. Induction of MOF formation by neonatal DES or E_2 treatment was also observed in this study (Table 3), consistent with previous reports (20, 21). Not surprisingly, given their relative potencies in binding to the estrogen receptor (73, 74), DES had a more pronounced effect than estradiol on follicle development.

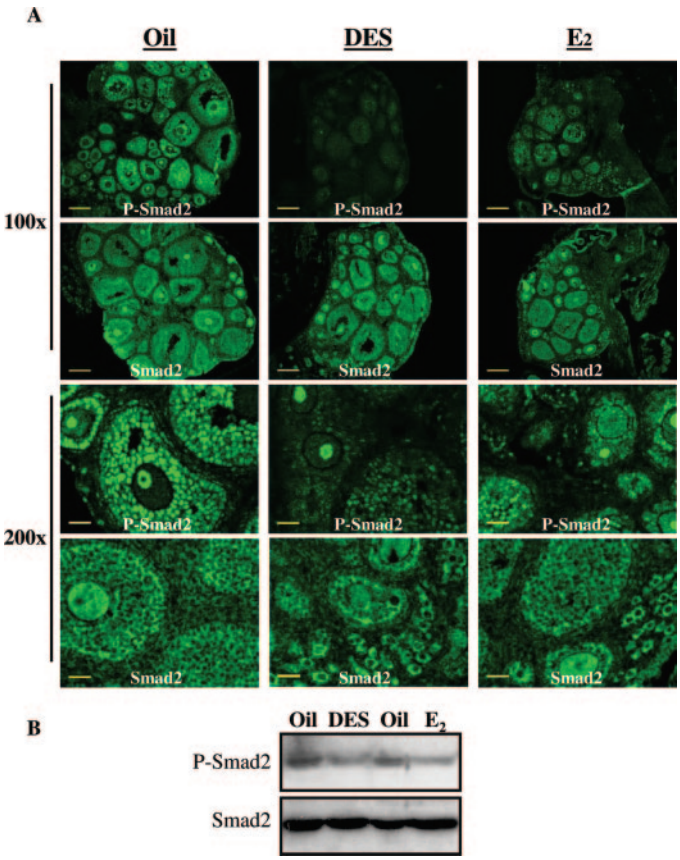

FIG. 4. Immunohistochemical and Western blot analysis of P-Smad 2 and Smad 2 expression in mouse ovaries after neonatal oil, DES, or E_2 treatment. A, Representative pictures from immunohistochemical studies. Experiments were repeated three times with three to four ovaries in each experiment. *Scale bars* in ×100 pictures, 100 μm. *Scale bars* in ×200 pictures, 20 μm. B, Representative Western blot pictures. Experiments were repeated twice. An average of quantitative results from the replicate experiments shows that, in DES- and E_2-treated mice, P-Smad2 protein levels are 54 and 60%, respectively, of those from the oil controls.

Discussion

Estrogen is important for ovarian follicle development and function, and estrogen treatment can impact a variety of target genes in the ovary. Our data reveal that neonatal DES or E_2 exposure hinders follicle development, decreases activin β-subunit mRNA and βA protein levels, and attenuates activin signaling in the ovary. Consistent with decreased β-subunit expression, serum inhibin levels are also decreased, which in turn likely causes increases in FSH. In addition, we show that estrogen can suppress activin subunit gene expression at a gene transcriptional level. Therefore, our study demonstrates that the activin genes are targets of estrogen action in the mouse ovary.

Activin and inhibin have been shown to play autocrine/paracrine roles in regulating ovarian follicle development (3–6), and regulation of activin and/or activin signaling component expression by estrogen has been suggested by several other studies. In the ovaries of 23- to 25-d-old rats treated with DES, although inhibin α-subunit mRNA levels were unaffected, both βA and βB subunit mRNA levels were strongly suppressed in isolated granulosa cells (75). In another study, E_2 treatment decreased βB subunit mRNA levels in ewe pituitary cells, whereas follistatin mRNA levels were not changed (51). In cultured MCF-7 human breast cancer cells, 10 nM E_2 treatment at different time points also suppressed βB subunit gene expression (52) and this finding was confirmed by a recent expression microarray study of MCF-7 cells treated with 100 nM E_2 for 6 and 12 h (53). These results are consistent with our findings. However, there is also evidence that estrogen can suppress ACTRII expression in rat hypothalamus (54) and increase follistatin expression in chicken granulosa cells (55), effects which were not observed in this study in the mouse ovary. In addition, an increase instead of a decrease in βB subunit gene expression in the uterus of neonatal estradiol valerate-treated sheep has been reported (56). When cultured immature rat granulosa cells were treated with 10 μM E_2 for 48 h, an increase in inhibin α and βB subunit mRNA levels was observed, whereas the

TABLE 3. Ovarian weight and follicle counts

Treatment	Ovarian weight (mg)	Follicle counting							
		Total	Primordial	Primary	Secondary	Small antral	Large antral	Atretic	MOF
Oil	1.69 ± 0.09^a	5606 ± 280^a	4381 ± 289	671 ± 51	398 ± 22	123 ± 25^a	18 ± 7	16 ± 11	0.2 ± 0.2^a
DES	0.78 ± 0.15^b	4368 ± 617^b	3135 ± 511	677 ± 111	518 ± 60	30 ± 5^b	7 ± 6	1 ± 1	38 ± 4^b
E_2	0.54 ± 0.01^b	5702 ± 323^a	4335 ± 450	820 ± 95	480 ± 50	60 ± 5^b	5 ± 3	5 ± 2	8 ± 1^b

Data are presented as mean ± SEM. n = 7–11 animals for MOF counting and n = 3–4 animals for the rest. *Different superscripts* within each measurement/counting category indicate statistical significance. $P < 0.01$ for ovarian weight; $P < 0.001$ for MOF counting; $P < 0.05$ for the rest.

βA subunit mRNA level was not altered (57). These differential effects of estrogen in regulating activin and/or activin signaling component expression suggest specificities in estrogen action among species and tissues, or related to the time and dose of estrogen treatment. Biphasic effects of estrogen depending on the time and dose have been documented in many systems (76–81).

In the present study, we also examined ovaries on d 6 immediately after neonatal estrogen treatment. We found that the mRNA levels of βA and βB subunits after DES treatment, and the mRNA levels of the βB subunit after E_2 treatment were decreased to the same extent in the d-6 ovaries as in the d-19 ovaries (data not shown). These results support the idea that a prolonged suppression in βA and βB subunit gene expression is most likely triggered during the neonatal estrogen treatment period and persists at least until postnatal d 19. Persistent changes in gene expression induced by prenatal or neonatal estrogen exposure have been demonstrated previously by microarray studies of mouse testis collected on postnatal d 21, 105, and 315, from which 32 genes have been identified as being altered in the long-term by the early estrogen exposure (58). In the current study, it is possible that estrogen treatment during the neonatal period impacts a subset of follicles at a certain critical developmental stage, and as those follicles develop and mature, they continue to express less activin. The persistent decrease in activin expression may also relate to the decreased number of small antral (and antral) follicles in the neonatal estrogen-treated animals. The latter explanation is supported by our results showing that ovary size as well as small-antral and antral follicle numbers are decreased in the neonatal DES- or E_2-treated mice. Our observations on the ovary size and follicle populations indicate a delay in follicle development in the DES- or E_2-treated animals, which is consistent with reports by others (18, 19).

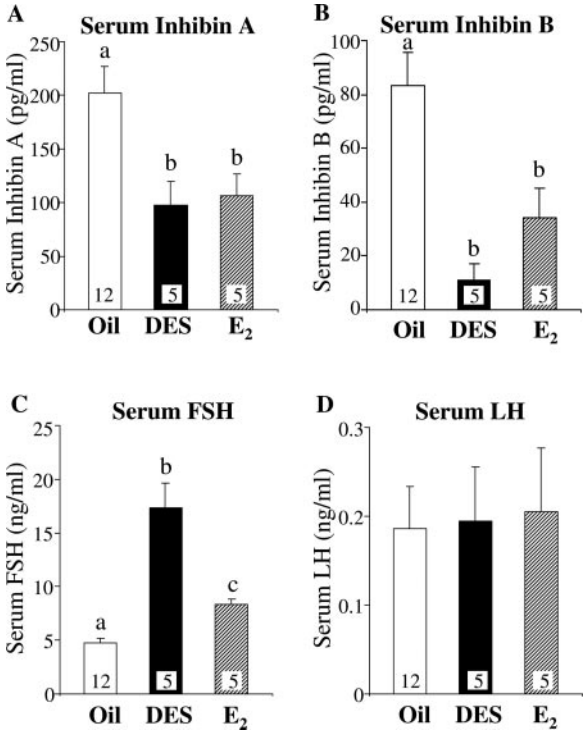

FIG. 5. Serum concentrations of inhibin A (A), inhibin B (B), FSH (C), and LH (D) measured in mice treated neonatally with DES or E_2. The *numbers at the bottom of each bar* indicate sample numbers. *Different lowercase letters above the bars* indicate statistical significance at $P < 0.01$.

It has been previously shown that FSH levels are actually decreased on d 6 and 12 in neonatal DES-treated mice compared with the controls, and an increase in FSH is subsequently observed at d 21 (72), suggesting that FSH levels are not chronically elevated in those animals. Our data collected on d 6 and 19 are consistent with these observations. Because inhibin is a potent suppressor of FSH synthesis and secretion (24, 25, 30), the elevated FSH levels on d 19 are likely caused by decreased serum inhibin A and inhibin B levels. This increase in FSH is not observed at d 6, perhaps because the decrease in FSH levels results from a negative feedback mechanism triggered by high estrogen levels immediately after the 5-d neonatal estrogen treatment. Inhibin is unlikely to contribute significantly to this early decrease in serum FSH levels on d 6, because it is thought that activin predominates in the early follicles, and inhibin increases in recruited follicles when FSH induces expression of the inhibin α-subunit in cells that have acquired the FSH receptor (3, 5, 38). Inhibin and activin can regulate FSH levels and, in turn, FSH can regulate expression of both inhibin α-subunit and β-subunits. It has been reported that when a 48-hr treatment with recombinant human FSH was given to cultured granulosa cells collected from 22-d-old rats, gene expression of inhibin α, βA, and βB subunits was significantly induced (82). In another study, FSH stimulates βA subunit production in cultured granulosa cells collected from 12-d-old rats (75). In our study, despite the significant elevation in serum FSH levels on d 19, activin subunit expression remained decreased in the ovaries from the neonatal estrogen-treated mice. Therefore, our results indicate that altered FSH is most likely not a direct cause of decreased activin expression, as it might be expected to actually increase activin gene expression.

The suppression of βA and βB subunit gene expression by neonatal estrogen exposure could be a direct effect of estro-

gen, because our data have revealed that E_2 significantly suppressed βA and βB subunit promoter activities in GRMO2 cells. Given that ERβ is the most abundant form of ER expressed in the granulosa cells (10, 11), one can speculate that the effect of estrogen on activin gene expression may be mediated by ERβ, although further studies are required to confirm this idea. Recently, genome-wide estrogen receptor binding sites have been analyzed in human MCF-7 breast cancer cells (53). Those binding sites often map more than 50 kb from proximal promoter regions of genes, and surprisingly, the proximal (1 kb) binding sites only constitute about 4% of all the binding sites (53). Therefore, it is possible that certain key estrogen receptor binding sites are not included in the promoter regions that we tested in this study, and this may explain why the observed suppression of activin subunit promoters by estrogen is relatively weak. Indeed, from the published mapping data (53), we found that estrogen receptor binding sites have been mapped within about 30 kb of the βA and βB subunit gene transcription start sites. It has also been reported that, in the 3′-untranslated region of the human activin βB gene, there is a potential estrogen response element (51). An indirect effect of estrogen through alterations in estrogen receptors, signaling cofactors, or other signaling pathways is also possible, and this requires further investigation.

In summary, the results from this study show that activin expression and signaling are regulated by estrogen. This study demonstrates that activin genes are targets for estrogen action in the early mouse ovary, indicating that some actions of estrogens might be mediated by changes in activin expression and signaling. Understanding how estrogen impacts activin signaling pathways in the ovary promises to increase our understanding of how these signals regulate normal follicle development and hence fertility.

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