The regulation of FSHβ transcription by gonadal steroids: testosterone and estradiol modulation of the activin intracellular signaling pathway

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Burger LL, Haisenleder DJ, Wotton GM, Aylor KW, Dalkin AC, Marshall JC. The regulation of FSHβ transcription by gonadal steroids: testosterone and estradiol modulation of the activin intracellular signaling pathway. Am J Physiol Endocrinol Metab 293: E277–E285, 2007. First published April 3, 2007; doi:10.1152/ajpendo.00447.2006.—Recent reports suggest that androgens and estrogens play a role in the regulation of FSHβ gene expression in the ovine pituitary. Androgens were reported to increase FSHβ mRNA in vitro (40) or in female mice carrying an ovine FSHβ transgene (24). In contrast, estrogen has been reported to suppress FSHβ gene transcription in ovine pituitary cells (3, 32, 38) and FSHβ mRNA in cultured female rat pituitary cells (7), and we (10) recently reported that estradiol (E2) suppressed FSHβ PT in castrate GnRH-deficient male rats.

The mechanism(s) whereby androgens and estrogens regulate FSHβ transcription is not well understood and may involve both classical steroid signaling and modulation of the signaling pathway for activin. Activin is a member of the TGF-β superfamily and is produced in a variety of tissues, including the gonadotrope, where it acts in a paracrine/autocrine manner to stimulate FSHβ transcription (4, 5, 48). Three to six androgen response elements (ARE) have been reported in the ovine and murine FSHβ promoters, and at least one is required for androgen action (41, 45). However, there are a number of reports that androgen may also regulate FSHβ transcription indirectly via activin. We reported that testosterone (T) rapidly and specifically increases FSHβ PT in GnRH-deficient male rats and was associated with a rapid decline in pituitary follistatin (FS) mRNA (10). FS is a glycoprotein, produced by both the gonadotropes and the pituitary folliculostellate cells, that binds to and neutralizes the bioactivity of activin (5). Additionally, others have reported that the effects of T on FSHβ mRNA either require activin or are blunted by FS (7, 30, 41).

The mechanism of estrogen action on FSHβ gene expression is less well understood. Miller and Miller (32) and Strahl et al. (42) have reported an estrogen-responsive region in the ovine FSHβ promoter, but the area does not contain an estrogen response element or bind estrogen receptor, suggesting that estrogen regulates FSHβ expression indirectly. There is some evidence that estrogens may also regulate FSHβ via an activin component. E2 increased pituitary FS PT and mRNA in female rats (26, 39), blunted the actions of activin on FS mRNA in female rat pituitary cells (7), and suppressed activin βB subunit mRNA in ovine pituitary cells (3).

Activin signals intracellularly via phosphorylation of Smad proteins. Briefly, activin binds to its type II receptor subunit, which then pairs with a type I receptor subunit, forming a heteromeric complex at the cell surface. Then serine/threonine kinase activity of the type II receptor phosphorylates the type I receptor, initiating a postreceptor signaling/phosphorylation cascade. The type I receptor phosphorylates Smad2 and -3. Phosphorylated Smad2 and/or -3 then partner with Smad4, and the complex translocates to the nucleus and binds DNA to initiate transcription.
regulate gene activity (for review, see Ref. 18). Additionally, activin intracellular signaling can be regulated by "inhibitory" Smad7, which binds to the type I receptor and prevents Smad2 or -3 phosphorylation or hastens their degradation via recruitment of phosphatases.

The objective of this study was to examine androgen and estrogen regulation of FSHβ gene transcription and whether it involves the availability of bioactive activin or the Smad intracellular signaling pathway. To this end, we characterized changes in activin βB mRNA, FS mRNA, Smad2, -3, -4, and -7 mRNA expression, and Smad2/3 phosphorylation in pituitaries from gonadectomized GnRH-deficient male and female rats treated with physiological levels of T and E2.

MATERIALS AND METHODS

Animal models. Adult (225–250 g) male and female Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used for all experiments. Rats were housed in a light- (lights on 0500–1700) and temperature-controlled (25°C) room and allowed access to food and water ad libitum. All surgeries were performed under isoflurane (2.5% isoflurane, balance O2; ISO-THESIA, Vetus Animal Health, Burns Veterinary Supply, Westbury, NY) anesthesia. Trunk blood was collected for serum hormone measurements. Pituitaries were collected and bisected, and then one-half was analyzed by RT-PCR and the Smads were determined by real time RT-PCR using an iCycler IQ (Bio-Rad, Hercules, CA) and the QuantiTech SYBR Green iCycler (Bio-Rad, Hercules, CA) and the QuantiTech SYBR Green iCycler (Bio-Rad, Hercules, CA). Assay conditions were optimized to generate a single PCR product as determined by melt curves and agarose gel electrophoresis. The primers used were activin βB forward (FWD) 5'-CCACCCGGATCAGTTTTAAT-3', reverse (REV) 5'-ACTCTACCTTCTGGTGATTAAGG-3', Smad2 FWD 5'-TTATCATC-CCAGAAAACACCA-3', REV 5'-CAAGGGCAGCTCCCCTT CCTCTA-3', Smad3 FWD 5'-GGGCTGCTAGAGCTTGTTGA-3', REV 5'-TGTTAGTCTACTCAGGGGGGA-3; Smad4 FWD 5'-GCAGATGTCTTACGGCGCTTCA-3, REV 5'-CGATCTCCTCCAGAAGGATCCA-3; and Smad7 FWD 5'-CAACCCCATACCTAGTTGCTGTA-3, REV 5'-CTGTGCTCTACCTTCTGATCCA-3. PCR product identity was confirmed by DNA sequencing. Unknown samples were measured using 10–100 ng RNA against an external standard curve. All samples, including standards, were measured in triplicate. All samples from a study were measured in the same assay. Mean intra-assay coefficients of variation are 12.1, 14.8, 12.8, 12.9, and 13.8% for activin βB and Smad2, -3, -4, and -7, respectively.

Pituitary FSHβ PT and activin βB, FS, Smad2, -3, -4, and -7 mRNAs, Smad phosphorylation, serum gonadotropins, and T were measured.

To determine whether T alters Smad phosphorylation in gonadotropes, we utilized the gonadotrope derived αT3 cells. αT3 cells were plated onto 32-mm culture dishes and grown until confluent in DMEM (containing phenol red, l-glutamine, 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Twenty-four hours before androgen treatment, the medium was replaced with phenol red-free DMEM with charcoal-stripped FBS (5% FBS). The next day, the medium was replaced with serum-free, phenol red-free DMEM for 4 h, and then cells were treated for 8 h with 100 ng/ml T or vehicle (0.2% ethanol; n = 6 wells/treatment). As a positive control for Smad phosphorylation, one to two separate wells were also treated with 30 ng/ml recombinant human activin A (R&D Systems, Minneapolis, MN) for 1 h. Upon completion, cells were washed with phosphate-buffered saline, lysed, and protein collected to determine Smad2/3 phosphorylation. Experiments were repeated at least two times to confirm results.

Because estrogen is the dominant gonadal hormone in females, we also investigated the effects of physiological levels of E2 on the regulation of FSHβ in female rats. Groups of female rats (n = 5–6/group) were ovariectomized (OVX) and then treated for 4 days with GnRH antagonist (100 μg LRF-147, 12 h). Rats then received one silicone implant containing E2 [25-mm column of 1 mg/ml E2 in sesame oil (Sigma); silastic tubing 1.6 mm id, 3.2 mm od (Dow Corning, Midland, MI)] that resulted in proestrus E2 levels (~50 pg/ml). Rats were then killed 0, 3, 8, and 24 h after the onset of E2 treatment. Pituitary FSHβ PT and activin βB, FS, Smad2, -3, -4, and -7 mRNAs, Smad phosphorylation, serum gonadotropins, and T were measured.

Measurement of serum hormones, RNA preparation, the mRNAs for activin βB, FS, and Smad mRNAs, and FSHβ PT. Serum LH and FSH were measured by RIA using the standards NIDDK RP-3 for LH and NIDDK RP-2 for FSH (National Hormone and Pituitary Program). The sensitivities for the LH and FSH assays are 0.09 and 0.8 ng/ml, respectively. The coefficients of variation are 4.7 and 13.5% (intra- and interassay) for LH and 4.6 and 14.4% for the FSH assay. T and E2 were measured by RIA using kits provided by Diagnostic Products (Los Angeles, CA) and Diagnostic Systems Laboratories (Webster, TX), respectively. The sensitivities and coefficients of variation for the assays are 0.1 ng/ml, 5.0%, and 8.2% (intra- and interassay) for T and 10 pg/ml, 5.2%, and 12.1% for E2.

Total pituitary RNA was extracted using the acid guanidium method (13). Residual genomic DNA was removed by treatment with 1 U RNase Free DNase I/pg RNA (Roche Molecular Biochemicals, Indianapolis, IN) at 37°C for 1 h. RNA preparations were confirmed to be DNA free by PCR in the absence of a preceding RT reaction. FSHβ PT and FS mRNA were measured by quantitative RT-PCR assays, as previously described (14, 28). The mRNA for activin βB and the Smads were determined by real time RT-PCR using an iCycler IQ (Bio-Rad, Hercules, CA) and the QuantiTech SYBR Green iCycler (Bio-Rad, Hercules, CA). The real-time assay for activin βB mRNA was performed on the amplicon from our previously described quantitative RT-PCR assay (8). The areas amplified for Smad2, -3, -4, and -7 mRNAs are based on previous real-time PCR assay reported by Drummond et al. (19). Assay conditions were optimized to generate one single PCR product as determined by melt curves and agarose gel electrophoresis. The primers used were activin βB forward (FWD) 5'-CCACCCGGATCAGTTTTAAT-3', reverse (REV) 5'-ACTCTACCTTCTGGTGATTAAGG-3', Smad2 FWD 5'-TTATCATC-CCAGAAAACACCA-3', REV 5'-CAAGGGCAGCTCCCCTTCTCTA-3', Smad3 FWD 5'-GGGCTGCTAGAGCTTGTTGA-3', REV 5'-TGTTAGTCTACTCAGGGGGGA-3; Smad4 FWD 5'-GCAGATGTCTTACGGCGCTTCA-3, REV 5'-CGATCTCCTCCAGAAGGATCCA-3; and Smad7 FWD 5'-CAACCCCATACCTAGTTGCTGTA-3, REV 5'-CTGTGCTCTACCTTCTGATCCA-3. PCR product identity was confirmed by DNA sequencing. Unknown samples were measured using 10–100 ng RNA against an external standard curve. All samples, including standards, were measured in triplicate. All samples from a study were measured in the same assay. Mean intra-assay coefficients of variation are 12.1, 14.8, 12.8, 12.9, and 13.8% for activin βB and Smad2, -3, -4, and -7, respectively.

Pituitary protein preparation Western immunoblot assays. For protein isolation, hemi pituitaries were homogenized in tissue lysis buffer [50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% NP-40, and protease and phosphatase inhibitor cocktails (P8340 and P5726, respectively, with stocks considered ×100; Sigma, St Louis, MO)]. Pituitary protein lysates (50 μg/rat sample, 30 μg/αT3 cell lysate) were resolved by electrophoresis (12.5% SDS PAGE) and then transferred to nitrocellulose filters. Receptor-mediated Smad2 and -3 (phosphorylated and total) proteins were detected with Western immunoblot assay. For phosphorylated Smad2 and -3, primary antibodies (rabbit) were obtained from Cell Signaling Technology (Beverly, MA); each antibody recognizes a protein of 58 kDa apparent molecular weight. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit (Upstate Biotechnology, Lake Placid, NY). As a positive control for Smad2 and -3 phosphorylation, each filter included two lanes containing 10–30 μg protein of αT3 cells that were either untreated or treated with activin A (30 ng/ml) for 1 h. Immunoactivity was detected using the Super Signal Pico West
The effects of T in male and female rats on pituitary FSHβ PT, activin βB mRNA, and FS mRNA are shown in Fig. 1. T administration induced a rapid (<3 h) and sustained (through 24 h) increase in FSHβ PT in both male and female rats, with the greater magnitude of increase (3-fold) in males vs. females (2-fold). Activin βB mRNA levels tended to decline after T in both sexes but did not reach significance. As previously reported (10), the increase in FSHβ PT in male rats was accompanied by a 50–60% reduction in FS mRNA. However, FS mRNA did not change after T in females.

The effects of T on Smad mRNAs are shown in Fig. 2. T increased the mRNAs for Smad2, -4, and -7 at 8 h (vs. 0-h controls) in male rats but had no effect on Smad mRNA expression in female rats. The effects of T on FSHβ PT in males have previously been shown to be androgen specific (10), and the effects of the nonaromatizable androgen DHT on female rats are shown in Table 1. Twenty-four hours of DHT increased serum FSH, FSHβ PT, and the mRNAs for Smad3, -4, and -7 and suppressed FS mRNA at 24 h. The differences in results between T and DHT in females are likely due to androgen dosage.

To determine whether T modulates Smad activity, we used Western immunoblot assays to measure pSmad2 and -3 as well as tSmad2/3, and results are shown in Fig. 3. In light of the rapid induction of FSHβ PT by T (2- to 3-fold increase <3h), we measured Smad phosphorylation between 0 and 8 h. At baseline, pSmad3 was barely detectable in male or female pituitary protein samples, and there did not appear to be any change with T treatment (Fig. 3A). In contrast, pSmad2 signal was easily detected and showed a significant induction by T. Between 3 and 8 h, pSmad2 increased 10- and sixfold, respectively, in male pituitaries, whereas in females the increase was not as large (1.5- to 2.5-fold) and peaked later at 8 h. T induced a small and transient increase in tSmad2/3 in female rat pituitary protein samples, and there did not appear to be any change with T treatment (Fig. 3A).
pituitaries at 3 h and had no effect on males. To control for protein loading and transfer, GAPDH levels were also measured for all blots, and no differences were observed between samples.

To determine whether androgen-induced increases in Smad phosphorylation occur in gonadotropes, we treated 

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T3 cells with 100 ng/ml T for 8 h. Similarly to results in rats, T increased Smad2 phosphorylation 3.9-fold vs. vehicle-treated controls after 8 h

\[ (P < 0.05 \text{ vs. controls}; \text{Fig. 4}) \]

. As in whole pituitary lysates, pSmad3 protein was very low basally and did not change with androgen treatment (results not shown). tSmad2/3 levels were also constant after T treatment.

**Regulation of the activin-signaling cascade by estrogen.** GnRH antagonist suppressed the post-OVX increase in serum LH, and neither serum LH nor FSH was affected by E2 treatment

\[ \text{FSH} = 4.9 \pm 0.5 \text{ ng/ml at 0 and } 24 \text{ h, respectively; FSH} = 5.4 \pm 0.4 \text{ ng/ml at 0 and } 24 \text{ h, respectively}. \]

Silicone implants containing E2 resulted in proestrus serum E2 levels (50.0 ± 5.0 pg/ml at 24 h). The effects of E2 on FSHβ PT, activin βB, FS, and Smad mRNAs are shown in Fig. 5. In contrast to prior data in male rats (10), E2 had no effect on pituitary FSHβ PT levels in females. The effects of E2 on activin βB and FS mRNA were biphasic. Activin βB mRNA increased 150% at 3 and 8 h before returning to control levels, whereas FS mRNA was suppressed 70% at 3 h before increasing to 150% of controls after 24 h. Smad2, -3, and -7 mRNAs were increased between 3 and 8 h and were transient for Smad2 and -7, but the rise in Smad3 mRNA was sustained through 24 h (Fig. 5). As with the previous experiments, pSmad3 was barely detectable and did not change after E2 (Fig. 6). pSmad2 increased two- to threefold 3–8 h after E2, and tSmad2/3 increased slightly at 8 h (Fig. 6). To control for protein loading and transfer, GAPDH levels were also measured for all blots, and no differences were observed between samples (data not shown).

**DISCUSSION**

The present results extend previous data and show that physiological T concentrations act directly on the pituitary to increase FSHβ transcription in both male and female rats. Furthermore, the effects of T are androgen specific, as nonaromatizable DHT increased FSHβ PT and E2 did not. Potentially, androgens could increase FSHβ transcription directly via the classical ligand-bound steroid receptor-signaling pathway, by enhancing the activity of other signaling systems (e.g., activin/Smad), or by a combination thereof. Spady et al. (41) and Thackery et al. (45) have reported that androgen increased the

\[ \text{Fig. 2. The time course of T action on pituitary Smad mRNAs in GnRH-deficient gonadectomized male and female rats. Experimental details are described in Fig. 1. All data are presented as %0–h (±SE) controls. Points with different letters are significantly different } (P < 0.05). \]

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<tr>
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<td>157.8±15.6</td>
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Data are presented as means ± SE. PT, primary transcript; FS, follistatin; OVX, ovariectomized; GnRH, gonadotropin-releasing hormone; T, testosterone; DHT, dihydrotestosterone. Serum FSH is presented in ng/ml and all others as %untreated (0–h) controls (n = 5–6/group). Means with different letters are significantly different P < 0.05.

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activity of ovine and mouse FSHβ promoter-reporter constructs transfected into pituitary-derived LβT2 cells. Additionally, they identified three and six AREs in the ovine and murine FSHβ promoters, respectively, and mutations in the most distal ARE in both, as well as most proximal ARE in the sheep, rendered the promoter constructs androgen insensitive. These data indicate that androgen acts, at least in part, through binding to its own receptor and interacting with androgen-responsive gene sequences to stimulate FSHβ transcription.

However, data reported here as well as in a number of earlier studies (7, 10, 30, 41) indicate that androgen may also regulate FSH indirectly via modulating the activity of activin. In male rats, the T-induced increase in FSHβ PT is temporally correlated with a suppression of FS mRNA, suggesting that T may also act indirectly by increasing the bioavailability of pituitary activin. Similarly, the administration of exogenous FS to pituitary cell cultures has been shown to block androgen-induced FSH secretion (7, 30). Also, Spady et al. (41) found that FS treatment or mutation of an activin-responsive Smad binding element (SBE) in the ovine FSHβ promoter abrogated the induction of the promoter by androgen. In contrast, our earlier studies in primary rat pituitary cells (10) revealed that, despite addition of exogenous FS, T treatment still increased FSHβ transcription. The differences between these two studies are likely due to experimental methodologies. Specifically, we cotreated rat pituitary cells with 30 ng/ml recombinant human FS androgen, whereas Spady et al. pretreated LβT2 cells for 20 h with 250 ng/ml rhFS before treatment with androgen ± FS. Therefore, in our paradigm the activin intracellular signaling system was likely undisturbed when cells were treated with androgen, which may account for why we still observed an increase in FSHβ PT, whereas they found that androgen-
induced FSHβ promoter activity was highly dependent on activin and presumably its intracellular signaling system. Accordingly, we aimed to determine whether androgen affected components of the activin-signaling system and whether those changes were correlated with FSHβ transcription.

We found that T rapidly increased FSHβ PT in both male and female rats but had sex-specific effects on FS and Smad mRNAs. In male rats, T suppressed FS mRNA and transiently increased the mRNAs for Smad2, -4, and -7. In contrast, in female rats a lower T dose increased FSHβ PT but had no effect on FS or Smad mRNA expression. The differences in FS and Smad mRNA expression between the sexes may reflect androgen dosage, as female rats treated with DHT responded in a fashion similar to males, with a larger increase in serum FSH and FSHβ PT, suppression of FS mRNA, and increased Smad3 and -4 mRNAs. However, it is important to note that in female rats T stimulation of FSHβ transcription occurs in the absence of androgen-specific changes in the activin-signaling pathway.

Because the changes in FS and Smad mRNAs are not required for androgen induction of FSHβ transcription, it is possible that androgens could modulate inhibitor Smad7 gene expression, which is known to be involved in negative feedback of activin intracellular signaling (18). Smad7 mRNA is present in both normal pituitary cells and gonadotrope cell lines and is stimulated by activin, a response that can be blocked by FS (5). Overexpression of Smad7 protein in LβT2 cells disrupts activin-induced increases in FSHβ gene expression (4, 20). However, we observed only modest changes in Smad7 mRNA concentration in male rats, and no differences were seen in females. Thus, alterations in the endogenous inhibitor of activin signaling, Smad 7, likely do not serve to modulate androgen action. There are no reports of hormone-modulated changes in Smad2, -3, or -4 mRNA in gonadotropes, but in other cell types TGFβ family members have been reported to increase these RNAs in a feed-forward mechanism to regulate the sensitivity of their signaling pathways (1). Overexpression of Smad3 alone or in combination with Smad4 increases FSHβ promoter activity in LβT2 cells (4, 20, 22, 29, 43, 44). Smad2 overexpression also increases FSHβ promoter activity, but only in combination with Smad4 (4, 20, 22, 29, 43). Additionally, FSHβ promoter activity is attenuated when Smad2 or -3 protein is suppressed (4, 20, 29).

Alternatively to changes in Smad gene expression, androgens could regulate the FSHβ gene via modulation of Smad (-2

Fig. 5. The time course of estradiol (E2) action on pituitary FSHβ-subunit PT and the mRNAs for activin βB, FS, and the Smads in GnRH-deficient ovariectomized (OVX) female rats. OVX rats (n = 4–6/group) were given GnRH antagonist every 12 h. Four days later rats received silastic capsules containing E2 and were killed 0, 3, 8, and 24 h later. All data are presented as %0–h (±SE) controls. Points with different letters are significantly different (P < 0.05).

Fig. 6. The effects of E2 on pSmad2 and tSmad2/3 in pituitary protein collected from GnRH-deficient OVX female rats. Changes in t- and pSmad2 immunostaining after E2 treatment were quantified by densitometry and expressed as %0–h (±SE) controls. Points with different letters are significantly different (P < 0.05).
and/or -3) phosphorylation. In pituitary cells, activin-induced increases in FSHβ transcription have been correlated to increased phosphorylation of Smad2 and -3 (4, 20). We investigated the effects of T on phosphorylation of the COOH-terminal SSXS motif of Smad2 and -3, which is regulated by the type I receptor. Despite data suggesting that Smad3, in combination with Smad4, plays the major role in transmitting the activin signal for the FSHβ gene (22, 43, 44), we were unable to detect pSmad3 signal either basally or after androgen exposure in pituitary lysates or αT3 cells. This did not appear to reflect limitations in our Western blot assay, as we had no trouble observing activin-induced Smad3 phosphorylation in αT3 cells. Therefore, we conclude that basal pSmad3 levels in the rat pituitary are low and unaffected by T. In contrast, pSmad2 levels were easily detected and increased after T administration; Smad2 phosphorylation increased more rapidly and to a greater degree in males vs. females, which again may reflect androgen dosage. One limitation of in vivo studies is that the pituitary is composed of multiple cell types, and it cannot be determined by Western blotting whether androgen induction of Smad2 phosphorylation is occurring in gonadotrope cells. To address this issue, we treated gonadotrope-derived αT3 cells with T and found that androgen also increases Smad2 phosphorylation in these cells, indicating that the changes we observed in vivo likely occur, at least in part, in the gonadotropes.

It is unusual to report that T increases just Smad2 phosphorylation and not Smad3. Ordinarily, a stimulus (activin or TGFβ) acting through the type I receptors will increase phosphorylation of both Smad2 and Smad3. One explanation for our observation of an increase in pSmad2, but not pSmad3, may be pSmad3 Ab sensitivity. As noted earlier, it was difficult to detect basal pSmad3 protein in either pituitary or αT3 cell lysates. Because we are measuring relatively small increases in Smad phosphorylation, compared with Smad activation by activin, it is possible that our assay is not sensitive enough to detect Smad3 phosphorylation in response to T. Alternatively, T may increase Smad2 phosphorylation through some unknown mechanism. There are several reports of differential activation of Smad2 and -3 in both hepatic and renal cells, which may be dependent on cell cycle stage, intracellular, and/or extracellular matrix environment and may also be independent of TGFβ or activin signaling (31, 33, 37, 46). There also appears to be differential actions of Smad2 and -3 on FSHβ transcription. Both Bernard (4) and Suszko et al. (43) report that abrogation of Smad2 or -3, by RNAi, reduces both basal and activin-induced FSHβ promoter activity, but only depletion of Smad3 reduces the magnitude increase in FSHβ response to activin. Both authors interpret these findings to indicate that, although Smad2 may not be as important in activin-induced FSHβ transcription, it does play a role in maintaining basal FSHβ levels. Additionally, Lamba et al. (29) found that overexpression of Smad2 in combination with Smad4 increased FSHβ promoter-reporter activity in LβT2 cells and that a combination of all three Smads (Smad2, -3, and -4) acted synergistically to increase FSHβ promoter activity fivefold greater than Smad3 and -4 alone. They also identified Smad2 in the transcriptional complex as a heterotrimmer of Smad2, -3, and -4, bound to the SBE of the mouse FSHβ promoter, and hypothesized that the trimer containing Smad2, -3, and -4 may either recruit more diverse coactivators or enhance the affinity of these regulators to the FSHβ promoter when Smad2 is present in the complex (29). Therefore, T-induced Smad2 phosphorylation may increase FSHβ transcription, in part, by either augmenting basal transcription and/or acting synergistically with pSmad3 and Smad4.

It is known that Smad binding to the activin-responsive region of the FSHβ promoter may not be enough to stimulate transcription of the gene. Transcription factors such the bicontinuous homeodomain factor Pitx2 or the TALE homeodomain proteins Pbx1 and Prep1 have been reported to be important partners with the Smads in stimulating FSHβ promoter activity (2, 44). It is also possible that androgen receptor (AR) might partner with the Smads in regulating FSHβ transcription. In prostate cell lines, AR has been reported (12, 23, 27) to form protein-protein interactions with each Smad3 or Smad4 and to modulate either AR interacting with androgen-responsive DNA elements or Smads interacting with Smad-responsive regions of DNA. Of note, the activin-responsive region of the rodent FSHβ promoter that contains the SBE (~266/~269 bp; 29, 43, 44) is within a larger hormone response element (~274/~260 bp) that confers both androgen and progesterone sensitivity on the FSHβ promoter (34, 35, 45, 47). It remains to be seen whether AR, or other steroid receptors, acting through protein-protein interactions, can be part of and/or regulate the transcriptional complex that binds to the activin response region of the FSHβ promoter.

We also examined the effects of estrogen on FSHβ transcription in female rats. In female rats, E2 had no effect on FSHβ transcription, which contrasts with previous results, where E2 markedly suppressed FSHβ PT in male rats (10). The differences between the two studies may be due to sex differences but may also reflect estrogen dose. The current study used a physiological proestrus amount (~50 pg/ml), whereas in the prior study in male rats circulating E2 levels were supraphysiological at ~120 pg/ml. The effects of E2 on FSHβ transcription in vivo are likely indirect, since E2 does not stimulate FSHβ mRNA synthesis in female rat pituitary fragments or a murine FSHβ promoter-reporter construct in LβT2 cells (40, 45). E2 has been shown to decrease activin βB mRNA and increase pituitary FS mRNA and PT in female rats (15, 26, 39). Surprisingly, we found that E2 had a biphasic effect on both activin βB and FS mRNAs, increasing βB mRNA at 3 and 8 h and initially suppressing FS at 3 h before rebounding to 150% of controls by 24 h. Coincident with the increase in βB and decline in FS mRNAs was a sustained increase in Smad3 mRNA and transient increases in Smad2 and -7 mRNAs. Similar to males, Smad3 phosphorylation was extremely low and was unchanged after E2. In contrast, pSmad2 and Smad2/3 were increased after E2. As E2 did not alter FSHβ transcription, this suggests either that the actions on activin, FS, and Smad2 and -3 (all of which could increase FSH) may be balanced by stimulation of the inhibitory Smad7. Alternatively, the changes in Smad mRNAs and phosphorylation may be occurring in other pituitary cell types, as estrogen is known to act through the TGFβ pathway to modulate lactotrope growth and differentiation (17).

In conclusion, androgens rapidly increase FSHβ PT in both male and female rats. The effects of T on FSHβ transcription are androgen specific, as they can be reproduced by DHT but not E2, and are correlated with modest, but significant, changes in the activin-signaling system. However, although changes in
Smad mRNAs and protein phosphorylation may be part of and/or facilitate androgen action of FSHβ transcription, they are not required.

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