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Transforming Growth Factor β -Activated Kinase 1 Is a Key Mediator of Ovine Follicle-Stimulating Hormone β -Subunit Expression

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Abstract

FSH, a key regulator of gonadal function, contains a β -subunit (FSH β) that is transcriptionally induced by activin, a member of the TGF β -superfamily. This study used 4.7 kb of the ovine FSH β -promoter linked to luciferase (oFSH β Luc) plus a well-characterized activin-responsive construct, p3TPLuc, to investigate the hypothesis that Smad3, TGF β -activated kinase 1 (TAK1), or both cause activin-mediated induction of FSH. Overexpression of either Smad3 or TAK1 induced oFSH β Luc in gonadotrope-derived L β T2 cells as much as activin itself. Induction of p3TPLuc by activin is known to require Smad3 activation in many cell types, and this was true in L β T2 cells, where 10-fold induction by activin (2–8 h after activin treatment) was blocked more than 90% by two dominant negative (DN) inhibitors of Smad3 [DN-Smad3 (3SA) and DN-Smad3 (D407E)]. By contrast, 6.5-fold induction of oFSH β Luc by activin (10–24 h after activin treatment) was not blocked by either DN-Smad inhibitor, suggesting that activation of Smad3 did not trigger induction of oFSH β Luc. By contrast, inhibition of TAK1 by a DN-TAK1 construct led to a 50% decrease in activin-mediated induction of oFSH β Luc, and a specific inhibitor of TAK1 (5Z-7-Oxozeanol) blocked induction by 100%, indicating that TAK1 is necessary for activin induction of oFSH β Luc. Finally, inhibiting p38-MAPK (often activated by TAK1) blocked induction of oFSH β Luc by 60%. In conclusion, the data presented here indicate that activation of TAK1 (and probably p38-MAPK), but not Smad3, is necessary for triggering induction of oFSH β by activin.

Abbreviations

BMP, Bone morphogenetic protein; Ca-ActRIB, constitutively active activin receptor IB; DN, dominant negative; α GSU, α -glycoprotein subunit; oFSH β Luc, ovine FSH β -promoter linked to luciferase; JNK, c-Jun N-terminal kinase; MAPKKK, MAPK kinase kinase; SBE, Smad binding element; TAB, TAK1-binding protein; TAK1, TGF β -activated kinase 1

FSH IS ESSENTIAL for female folliculogenesis and stimulates spermatogenesis in males (1). It is an α/β heterodimer made only in pituitary gonadotropes where transcription of its unique β -subunit (FSH β) is paramount in determining overall FSH production. Transcription of FSH β is controlled primarily by GnRH (2,3) and gonadal- or pituitary-derived activins, inhibins, and follistatin (2–10).

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Activin, a major inducer of FSH β -transcription, seems central to FSH expression *in vivo*. It comprises three isoforms that are homo- or heterodimers of activin β A or β B chains (7). Activin A (homodimer of β A subunits) is the most potent inducer known for FSH β (11), although other members of the TGF β family have been shown to induce transcription of the FSH β -subunit (5,8). Recent studies suggest that activin action not only induces FSH β but is also important for GnRH-mediated induction of FSH β expression as well (12,13).

Activin exerts its biological effects by binding to activin type II serine/threonine kinase receptors (ActRII or ActRIIB), which then bind and phosphorylate the activin type I receptor (ActRI, also known as ALK4) (14–20). Once this occurs, the signal can be transmitted downstream by phosphorylating and activating Smad2 and/or Smad3. One or more of these Smads associate with a common signaling molecule, Smad4 (Co-Smad), to form a Smad2/4 or Smad3/4 complex that translocates to the nucleus, where it binds a specific DNA sequence known as a Smad binding element (SBE). The affinity of Smad complexes for DNA is low, but binding is usually stabilized by interaction(s) with adjacent tissue- and cell-specific transcription factors (21–28).

Individual or palindromic SBEs have been identified in both ovine and rat FSH β -promoters, and they appear to be important for activin-mediated induction of FSH β . Several are reported to bind Smad4. Also, the tale homeodomain of Pbx1 and Prep1 has been shown to partner with Smad4 within a DNA binding complex in the ovine FSH β -promoter, and the same was shown for Pitx2c in the rat FSH β -promoter (29,30). Furthermore, overexpression of Smad3 (but not Smad2) has been shown to increase basal and activin-induced expression of FSH β in L β T2 cells. Nevertheless, the binding of Smad4 to SBEs in the ovine and rat promoters of FSH β is not significantly increased after treatment of cells with activin (29–31). Therefore, whereas Smad4 and its associated transcription factors appear to play pivotal roles in expression of FSH β , no data actually prove that activin triggers FSH β induction through the activation of Smad3 and subsequent binding to Smad4 and one or more FSH β -SBEs.

Recently a Smad-independent pathway has been associated with the actions of several TGF β family members [TGF β , bone morphogenetic proteins (BMPs), activins] that use TGF β -activated kinase 1 (TAK1), a member of the MAPK kinase kinase (MAPKKK) family (32). Activation of TAK1 requires the association of TAK1-binding protein (TAB)1, which induces a conformational change in TAK1, allowing it to autophosphorylate its activation domain (33). The activated TAK1/TAB1 complex also binds TAB2 or TAB3 proteins, which modulate the regulatory effects of TAK1/TAB1 (34–38). Activation of TAK1 initiates MAP kinase cascades that have been shown to phosphorylate downstream c-Jun N-terminal kinase (JNK), ERK, and p38 (34,39–46).

The studies presented here focused initially on the hypothesis that Smad3 induces expression of an ovine FSH β -promoter-luciferase construct (oFSH β Luc) that contains all the regulatory sequences needed for normal cell-specific expression and regulation of FSH β in mouse gonadotropes (47–50). The transformed gonadotrope cell line L β T2 was used as a model to study the signaling pathway mediating activin induction of FSH β expression (51). Studies included comparisons with a well-characterized activin-responsive construct known to require Smad3 for induction, p3TPLuc (48). When it appeared that Smad3 was important for basal expression of oFSH β but could not be proven to trigger activin-mediated induction of oFSH β Luc, the studies focused on the hypothesis that a novel Smad-independent pathway uses TAK1 activation to induce oFSH β Luc.

Materials and Methods

Reagents

Recombinant human activin A was obtained from R&D Systems, Inc. (Minneapolis, MN). MAPK inhibitors SB203580, SP600125, and PD98059 were purchased from Calbiochem (San Diego, CA). Recombinant human follistatin 288 was obtained from Dr. A. F. Parlow at the National Hormone and Pituitary Program (Torrance, CA), and 5Z-7-Oxozeanol (TAK1 inhibitor) was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Reporter plasmids and expression vectors

The ovine reporter plasmid oFSH β Luc has been previously described (52). Smad3 and dominant negative (DN) Smad3 [DN-Smad3 (D407E)] were obtained from Dr. Mitsuyasu Kato (University of Tsukuba, Tsukuba, Japan) (53). DN-Smad3 (3SA) was obtained from Dr. Theresa A. Guise (University of Texas, San Antonio, TX) (54). Expression constructs for TAK1 (55), TAB1 (55), TAB2 (36), TAB3 (34), and DN-TAK1-KN (56) were obtained from Dr. Jun Tsuji (North Carolina State University, Raleigh, NC). Constitutively active activin receptor IB (Ca-ActRIB) was obtained from Dr. J. Wrana (Mount Sinai Hospital, Ontario, Canada) (18). Mock plasmid (pCMV DNA), containing the CMV promoter with no luciferase reporter gene, was obtained from Promega Corp. (Madison, WI). Control plasmid (pGL3-control) containing the GL3 luciferase reporter driven by an SV40 promoter was purchased from Promega.

Culture and transient transfection of L β T2 gonadotropes

L β T2 cells (51) were grown at 37 C in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin G, and 100 μ g/ml streptomycin under 95% air-5% CO₂. Cells were grown in 150-cm² flasks until they were confluent and then transferred to 96-multiwell plates (Falcon; Fisher Scientific, Raleigh, NC) at a concentration of 25,000 cells/well. Cells were cotransfected, 24 h later, in triplicate with 50 ng oFSH β Luc, p3TPLuc, pGL3-control and 50–150 ng pCMV using Fugene6 (Roche Molecular Biochemicals, Basel, Switzerland). After transfection (24 h), cells were treated with fresh media, with or without activin (100 ng/ml), for an additional 24 h. Cells were then lysed in 50 μ l passive lysis buffer, and 20 μ l was assayed for luciferase activity.

Real-time RT-PCR

Total RNA from L β T2 cells was isolated and converted to cDNA as reported (5). Oligonucleotides for taqman real-time PCR were designed for mouse FSH β , LH β , and the α -glycoprotein subunit (α GSU) common to both LH and FSH, using software from Integrated DNA Technologies, Inc. (Coralville, IA). Mouse 18s ribosomal RNA served as the internal control for quantifying all molecules as reported earlier (57). The taqman probe for FSH β was reported previously (57), and the primers and probes for LH β and α GSU were: LH β forward, 5'-AATCCCGCTCCA-CACAGTACATGA-3'; LH β reverse, 5'-TCAGCTCAGGAGGTGTC-CATTGTT-3'; LH β probe, 5'-TGCCTTCCTTCAATGAGCTCAAAG-GCGA-3'; α GSU forward, 5'-AGATCGACAATCACCTGCCAGAA-3'; α GSU reverse, 5'-AGGAACATGGACAGCATGACCAGA-3'; α GSU probe, 5'-TCCAGAGCTTGCAGAAGAGCTATGGA-3'.

Real-time PCR was performed in duplicate on triplicate cDNA samples from L β T2 cells using an i-Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Samples were incubated at 95 C for 3 min, and then for 40 complete cycles (95 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec).

There was a final extension step of 72 C for 3 min. Threshold cycle values were determined with Bio-Rad software and used for relative quantization with the $2^{-\Delta\Delta C_t}$ method.

Luciferase assay

Cells were harvested by adding $1 \times$ passive lysis buffer (Promega, Madison, WI) as described above for L β T2 cells. Luciferase activity was measured in 40% of the cell lysate (20 μ l) by adding 100 μ l luciferase assay system from Promega. Luciferase activity was measured for 20 sec using a Monolight 2010 single tube luminometer (Analytical Luminescence Laboratory, San Diego, CA) or automated Victor-Light microplate luminometer (PerkinElmer, Boston, MA).

Antibodies

The rabbit antimouse TAK1 (554–579) and rabbit antimouse TAB1 (480–500) polyclonal antibodies (Upstate Biotechnology, Inc., Lake Placid, NY) were also used to detect endogenous TAK1 and TAB1 in L β T2 cells. Secondary antibody used for detection was antirabbit IgG (Calbiochem).

Western blotting

Analysis of TAK1 and TAB1 expression by Western blot was performed as previously described (55). Briefly, L β T2 cells were cultured in 6-well tissue culture plates with 1 million cells/well for 24 h. Cells were pretreated with follistatin-288 (250 ng/ml) for 16 h to minimize FSH β expression (unpublished results by Dr. H. J. Huang, this laboratory), presumably by eliminating any effects of endogenously produced activin or activin-like activin (5). Cells were then washed and treated with or without activin A (100 ng/ml) for the indicated time points. Cells were washed once with PBS, pH 7.4 (PBS; 4 C) and collected in 1 ml PBS. Cells were centrifuged at $1000 \times g$ for 5 min (4 C) and lysed in 50 μ l of 0.5% Triton X-100 lysis buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 12.5 mM β -glycerophosphate; 1.5 mM MgCl $_2$; 2 mM EGTA; 10 mM NaF; 2 mM dithiothreitol; 1 mM sodium orthovanadate; 1 mM phenylmethylsulfonylfluoride; and 20 mM aprotinin). Cells were sonicated for 5 sec and centrifuged at $10,000 \times g$ for 5 min, and the cleared lysates were fractionated on a 7% SDS-polyacrylamide gel. Proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated with antibodies, and antibody localization was visualized with horseradish peroxidase-conjugated antibodies to rabbit IgG using the enhanced chemiluminescence Western blotting system (ECL; Amersham).

Statistics

Data (see Fig. 7; Western blot) were obtained two times, and the clearest blot is shown. Data from all other experiments were replicated at least three times, and all samples were assayed in triplicate. Means \pm SEM values are shown in all figures; data in all (with one exception; see Fig. 7) were analyzed using one-way ANOVA with Tukey's multiple comparison test according to the Prism version 4 (GraphPad Software, Inc., San Diego, CA).

Results

Transfected Smad3 increased basal and activin-induced expression of oFSH β Luc equally

To investigate the role of Smad3 in mediating oFSH β induction by activin, L β T2 cells were cotransfected with oFSH β Luc and increasing amounts of Smad3 DNA (25, 50, 75, 100, and 125 ng) (Fig. 1). Cotransfection with Smad3 increased basal expression of oFSH β Luc by 89, 116, 206, 240, and 312%, respectively. Activin treatment of cultures with transfected Smad3 increased oFSH β Luc induction above control cultures. However, increased induction paralleled increased basal expression at all concentrations of transfected Smad3, with an

average increase of 4.5 ± 0.3 . The ratios of induced/basal expression at each level of Smad3 were: 4.6 ± 2.1 , 5.6 ± 1.5 , 4.2 ± 1.1 , 4.6 ± 1.2 , and 3.7 ± 1.0 , respectively, for the 25- to 125-ng treatments. None of these were significantly different from each other, nor were they different from the 5.7 ± 1.6 ratio observed in control cultures not transfected with Smad3 (Fig. 1).

Smad3 activation is required for induction of p3TPLuc but not for oFSH β Luc

To determine the functional significance of endogenous Smad3 signaling, two DN inhibitors of Smad3 [Smad3 (3SA) and Smad3 (D407E)] were tested. First, L β T2 cells were co-transfected with p3TPLuc, which can be induced by activin through a Smad3 pathway. Then p3TPLuc was cotransfected with one of two DN-Smad3 expression vectors to block the actions of endogenous Smad3 (see Fig. 2) (49,53). Basal expression of p3TPLuc was not inhibited by either DN-Smad, but both inhibitors blocked 7-fold induction by activin by 87–96%. These data showed that DN-Smad3 (3SA) and DN-Smad3 (D407E) were effective inhibitors of activin-mediated activation of Smad3 in L β T2 cells.

Data (see Fig. 2B) show that DN-Smad3 (3SA) or DN-Smad3 (D407E) did not block basal or activin-mediated induction of oFSH β Luc. Activin induced oFSH β Luc expression by 5.1-fold, but neither Smad3 inhibitor altered the action of activin.

Subsequent results (see Fig. 2C) are similar, except that Ca-ActRIB was used to induce oFSH β Luc in L β T2 cells. The results show that cotransfection of Ca-ActRIB along with oFSH β Luc increased expression of oFSH β Luc by 9.5-fold. The DN inhibitors of Smad3, however, had no effect on this induction, just as observed for induction of oFSH β Luc with activin. These data show that the constitutively active activin receptor induced oFSH β Luc but that activation of Smad3 was not necessary for induction to occur.

Data indicate (see Fig. 2D) that there was no tendency for DN-Smad3 (3SA) to inhibit either basal or activin-mediated induction of oFSH β Luc over a range of concentrations (25–150 ng) higher and lower than the 50-ng amount used in Fig. 2, A–C. Activin increased expression of oFSH β Luc by 5.8-fold over basal expression, with or without DN-Smad3 (Fig. 2D).

Activin induction of oFSH β Luc and p3TPLuc have very different kinetics

The data in Fig. 3 show time-course data (24 h) for activin induction of oFSH β Luc and p3TPLuc. In Fig. 3A, activin A (100 ng/ml) had no observable effect on oFSH β Luc expression for 6 h. It then had a small, inductive effect from 8–12 h (~100% increase) before it increased expression linearly up to full induction at 22 h (520% increase). When DN-Smad3 (3SA) was cotransfected, the same pattern was observed, and there was no inhibition by DN-Smad3 (3SA) at any time during the 24-h period.

The data in Fig. 3B show that p3TPLuc was induced 6-fold at 2 h and 9-fold at 6 h and dropped to 2-fold by 10 h. Therefore, induction by activin was initiated well before 2 h and down-regulated shortly after 8 h. When cultures were cotransfected with DN-Smad3 (3SA), most of the induction between 2–10 h was blocked. Results shown with the DN-Smad3 in this time-course format indicated that activin induced expression of oFSH β through a pathway that did not depend on Smad3 activation.

Altering expression of oFSH β Luc with TAK1, TAB1, TAB2, TAB3, and DN-TAK1

Next, the role of TAK1 as a potential mediator of activin induction was tested. L β T2 cells were transfected with either oFSH β Luc alone or with oFSH β Luc plus TAK1 and its binding proteins, TAB1, TAB2, or TAB3. Preliminary studies showed that overexpression of TAK1/TAB1 induced oFSH β Luc 45-fold, but this appeared to be non-specific induction because it also

increased expression of the SV40-driven pGL3Luc control construct and the minimal thymidine kinase luciferase construct, T109Luc, that are not activin responsive (data not shown). However, cotransfection of TAK1 with either TAB2 or TAB3 did increase expression of oFSH β Luc, just like activin (6.3-fold), and activin was unable to augment this induction much above 6.3-fold when added along with TAB2 or TAB3. Thus, transfections with TAK1 and either TAB2 or TAB3 nearly substituted for activin induction of oFSH β Luc (Fig. 4A), suggesting they could be in the signaling pathway used by activin.

Transfections with TAK1/TAB2 or TAK1/TAB3 were tested with the pGL3Luc control expression vector, and the data in Fig. 4B show that its expression was not altered. Thus, overexpression of TAK1 in the presence of TAB2 or TAB3 was specific for inducing activin-responsive genes only.

To determine whether endogenous TAK1 was important for activin action, L β T2 cells were cotransfected with oFSH β Luc and a DN TAK1 mutant (DN-TAK1), which lacks the active site required for its kinase activity (32). This DN molecule (DN-TAK1) did suppress activin induction of oFSH β Luc by 50% (Fig. 4C). This finding was consistent with the concept that endogenous TAK1 is, at least partly, responsible for activin-mediated induction of oFSH β .

Inhibiting activin induction of oFSH β Luc with a TAK1 inhibitor (5Z-7-Oxozeanol)

To further characterize the role of TAK1 in activin-mediated induction of oFSH β Luc, L β T2 cells were transfected with oFSH β Luc and then treated with increasing concentrations of 5Z-7-Oxozeanol, a newly discovered inhibitor of TAK1 that works by blocking the ATP binding site required for TAK1 kinase activity (55). Cells were treated with activin (100 ng/ml) and with or without 5Z-7-Oxozeanol for 24 h. Activin alone induced oFSH β Luc expression 11-fold, and addition of 5Z-7-Oxozeanol inhibited 100% of this induction in a dose-dependent manner, with an ED₅₀ of 1.2 μ M (Fig. 5A). There was no effect of 5Z-7-Oxozeanol on basal expression of oFSH β Luc (Fig. 5A).

The data in Fig. 5B are similar to those in Fig. 5A, but cotransfection of TAK1/TAB1, which constitutively activates TAK1, was used to induce oFSH β Luc instead of activin (Fig. 4A). The potency of 5Z-7-Oxozeanol was tested on L β T2 cells cotransfected with TAK1/TAB1 to compare the ED₅₀ values obtained for inhibition of endogenous TAK1. Expression of oFSH β Luc was increased 35-fold by TAK1/TAB1, and 5Z-7-Oxozeanol inhibited this induction by 99% in a dose-dependent manner, with an ED₅₀ of 1.5 μ M. As in Fig. 5A, basal expression of oFSH β Luc was unaffected by the chemical inhibitor. These results are consistent with the concept that 5Z-7-Oxozeanol strongly inhibited the extra TAK1 produced by transfecting L β T2 cells with a TAK1 expression construct.

The effects of TAK1 inhibitor 5Z-7-Oxozeanol were tested on the activin signaling pathway using Ca-ActRIB (T206D) to induce oFSH β Luc as in Fig. 2C. Cotransfection of Ca-ActRIB (T206D) stimulated oFSH β Luc by 8.4-fold (Fig. 5C), and addition of 5Z-7-Oxozeanol inhibited this expression by 95%, with an ED₅₀ of 1.6 μ M.

Specificity of 5Z-7-Oxozeanol inhibition

To confirm that 5Z-7-Oxozeanol was a specific inhibitor of oFSH β Luc, the effects of 5Z-7-Oxozeanol were tested on endogenous FSH β and the two other closely related gonadotropin subunit genes expressed in L β T2 cells. L β T2 cells were treated with activin, with or without 5Z-7-Oxozeanol, and total RNA was isolated and analyzed using real-time RT-PCR for mouse FSH β -mRNA (Fig. 6A). In addition, analyses were performed for α GSU subunit mRNA (Fig. 6B) and LH β mRNA (Fig. 6C). The data show that 24 h of activin treatment increased mRNA for mouse FSH β by 922-fold and that 5Z-7-Oxozeanol inhibited this induction by more than

99%. Although activin is often reported to be neutral toward expression of the α GSU, activin decreased mRNA for α GSU by 92% in this series of experiments, and 5Z-7-Oxozeanol reversed most of this inhibition. Likewise, mRNA for LH β was slightly inhibited by activin (30% inhibition), and 5Z-7-Oxozeanol reversed this inhibition, to create an actual increase in LH β mRNA of 60%. These data indicated that 5Z-7-Oxozeanol reversed the effects of activin on the expression of every glycoprotein hormone subunit produced in L β T2 cells. Because gene expression of α GSU and LH β were stimulated, it was clear that 5Z-7-Oxozeanol did not inhibit protein synthesis or cellular function in general.

TAK1 is present in L β T2 cells and is phosphorylated 2–24 h after activin treatment

To establish the existence of TAK1 in L β T2 cells and its time-course of phosphorylation by activin, L β T2 cells were pretreated with follistatin for 16 h to bionutralize any endogenously made activin and then treated with activin for 2–24 h. Cell extracts were prepared and analyzed by Western blot techniques to visualize TAK1, phosphorylated TAK1, and TAB1 simultaneously. The data in Fig. 7 show that L β T2 cells expressed both TAK1 and TAB1 abundantly. TAK1 migrated with a molecular mass of 70 kDa, and TAB1 migrated with a molecular mass of 55 kDa, as expected (33). The immuno-blot revealed that activin phosphorylated TAK1 between 1–2 h (Fig. 7). Activation of TAK1 by activin was greatest at 4, 6, and 8 h but was maintained at higher-than-control levels up to 24 h. There was no change in overall expression of TAK1 or TAB1 during activin treatment.

p38 MAPK participates in activin-mediated induction of oFSH β Luc

TAK1 is a member of the MAPKKK family that can phosphorylate and activate members of the MAPK family, including the ERK, JNK, and p38 MAPKs. To determine whether MAPKs are involved in activin-mediated induction of oFSH β , cultures of L β T2 cells were treated with or without specific chemical inhibitors of ERK, JNK, and p38 MAPKs with or without activin to determine whether the inhibitors would specifically block activin induction of oFSH β Luc expression. A chemical inhibitor of JNK (JNK II inhibitor) had no effect on induction of oFSH β Luc by activin (Fig. 8A). An ERK-specific chemical inhibitor (PD98059) did not block activin induction of oFSH β Luc, but it did increase basal expression, suggesting that ERK plays an inhibitory role in basal expression of the oFSH β -gene (Fig. 8B). Finally, a well-known inhibitor of p38 (SB203580) inhibited activin action by 60% (Fig. 8C). These results suggest that p38-MAPK is downstream of the TAK1 pathway and that activation of p38-MAPK is important for activin induction of oFSH β Luc.

Discussion

It is well known that activin can increase the synthesis (and associated secretion) of FSH by inducing transcription of its β -subunit (FSH β) (11,58). It is also known that activin classically transmits its signal intracellularly by phosphorylating Smad2 or Smad3, and it was recently reported that expression of extra Smad3 (but not Smad2) increases basal and activin-induced expression of rat (30) and ovine FSH β -subunits in L β T2 cells (29). Initial studies in our laboratory focused on the role of Smad3 as a potential mediator activated by activin; however, the data showed that activin is able to stimulate FSH β expression independent of Smad3 activation. The studies reported here identified that a TAK1 pathway is critical for mediating activin induction of FSH β expression in gonadotropes.

Transfection of Smad3 is known to increase basal expression of Smad-dependent genes to levels comparable with induction by activin (59). Preliminary studies showed that Smad3 overexpression stimulated both basal and activin-induced expression of the FSH β -gene. However, overexpression of Smad3 did not increase the ratio of induction/basal expression of oFSH β Luc over a wide range of Smad3 concentrations, including those that were maximally

effective. One interpretation is that overexpression of Smad3 simply stimulates basal expression of oFSH β Luc. If this is all that occurs, the increased induction by activin observed in the presence of extra Smad3 might occur simply because transcription started at a higher level. This explanation does not imply that activin works through the activation of Smad3. Therefore, the data in Fig. 1 fail to prove that Smad3 participates in activin-mediated induction of the FSH β -subunit.

In this report, it is shown, for the first time, that inhibiting activation of endogenous Smad3 does not affect activin's ability to induce expression of the FSH β -subunit. Two well-characterized Smad3 mutants [Smad3 (3SA) and Smad3 (D407E)] were used to block possible activation of endogenous Smad3 by activin. Smad3 (3SA) has three serines required for activation mutated to three alanines (60). Smad3 (D407E) has an aspartate mutated to glutamate, which allows it to bind tightly to the activin type I receptor's (ActRIB) active site, therefore blocking the activation of Smad2 and Smad3 signaling pathways (53). The results show that neither of these inhibitors blocked activin-induced expression of oFSH β Luc (Fig. 2, B–D). Furthermore, both DN inhibitors were shown to be fully effective in L β T2 cells, because they blocked the ability of activin to induce p3TPLuc, which is known to depend on Smad3 activation in other cell types (59). These data can only be interpreted to mean that activin does not require the activation of endogenous Smad3 to induce FSH β -gene. Furthermore, because DN-Smad3 (D407E) blocks activation of Smad2 along with Smad3 in other cell systems (53), its failure to block activin-mediated induction of oFSH β Luc strongly suggests that activation of neither Smad2 nor Smad3 is required for activin-mediated induction of FSH β -subunit expression.

The results were surprising because both rat and ovine FSH β -promoters contain consensus Smad binding sites (29,30) at positions that are important for activin-mediated induction, and data show Smad4 binding to several of these positions. However, it should be noted that previous publications using L β T2 cells have shown Smad4 binding is observed under basal conditions and is not enhanced due to activin treatment (61). The role of Smads in binding to FSH β -promoter elements is still unclear. It might be possible for Smads to play a role at the basal transcription complex independent of activin, where Smad3 or Smad4 are continuously moving into the nucleus to bind SBE(s) that drives basal transcription as shown in other systems (61,62). This remains an unsolved question of activin action.

Another important finding is the different activation kinetics observed between oFSH β Luc and Smad-dependent construct (p3TPLuc) in response to activin treatment. In this report, the time-course of activin induction of Smad-dependent p3TPLuc showed very different kinetics compared with induction of oFSH β Luc (Fig. 3). Induction of p3TPLuc was relatively rapid (2–8 h), whereas induction of oFSH β Luc was observed only after 8 h of treatment. Essentially all previous reports using FSH β Luc in L β T2 cells measured activin induction at 24 h (51), which matched the optimal time observed in the studies reported here. Because Smad2 and Smad3 are normally activated within 1–4 h (49), the data in Fig. 4 provide further evidence that neither Smad2 nor Smad3 is the immediate trigger for activin induction of oFSH β Luc. Based on kinetics, however, Smad3 could be the immediate trigger for induction of p3TPLuc. These data even suggest the possibility that oFSH β Luc induction depends on one or more late genes that might be under control of one or more early response genes affected by activin, but the early genes, themselves, should not depend on Smad3 for this action because DN-Smad3 inhibitors do not block activin induction of oFSH β Luc. Multigene analysis could be necessary to study this possibility further.

The evidence showing that endogenous Smad3 (or Smad2) is not the trigger for activin-mediated induction of oFSH β Luc led to experiments that explored a Smad-independent pathway involving TAK1 (63,64). Activin, TGF β , and BMP were shown in multiple studies

to signal through activation of TAK1 (MAPKKK), initiating a MAPK signaling cascade; therefore the initial results directed the focus of the study to the possible role of TAK1 in mediating activin stimulation of FSH β -gene. Initially, a series of experiments with TAK1 and TAB1 showed that their dual overexpression cause robust stimulation of the oFSH β Luc, as previously shown in other systems of TAK1 (55). However, overexpression of TAK1 along with TAB1 results in constitutive kinase activation of TAK1, resulting in activating other genes including the control constructs. TAB2 and TAB3 were also identified as TAK1 binding partners, but they differ from TAK1 by their ability to bind to upstream regulatory elements of the signaling pathway. It has been recently shown that TAK1 binds TAB1 and can either partner with TAB2, forming a TAK1/TAB1/TAB2 complex, or partner with TAB3 to form a TAK1/TAB1/TAB3 complex. Therefore, experiments with TAK1 and TAB2 and TAB3 showed that overexpression of TAK1 with either TAB2 or TAB3 specifically induced oFSH β Luc. Furthermore, this induction substituted almost entirely for activin, which is consistent with TAK1 being an essential member of the activin signaling pathway. In addition, it was found that a DN inhibitor of endogenous TAK1 blocked activin-mediated induction of oFSH β Luc by 50%. This, too, was consistent with a physiological role for TAK1 in oFSH β Luc expression.

Supplementary studies with a newly available TAK1 inhibitor, 5Z-7-Oxozeanol, established that TAK1 was required for activin-mediated expression of oFSH β . 5Z-7-Oxozeanol is a resorcylic acid lactone that is currently being investigated for its potential as a therapeutic agent for allergic cutaneous disorders. It has been shown to specifically inhibit the catalytic activity of TAK1 without affecting any of the other MAPKKK family members (55). The use of 5Z-7-Oxozeanol to inhibit endogenous TAK1 activation in L β T2 cells showed that: first, the TAK1 inhibitor was shown to fully block activin induction of oFSH β Luc in a dose-dependent manner (Fig. 5); then, it was shown that 5Z-7-Oxozeanol blocked induction of endogenous mouse FSH β -mRNA in L β T2 cells by 92%, and specificity was established by showing that 5Z-7-Oxozeanol did not decrease mRNAs for endogenous LH β and α GSU (Fig. 6). In fact, the TAK1 inhibitor actually increased levels of these latter mRNAs by reversing the effects of activin on these gonadotropin subunit mRNAs. Although regulation of FSH β in L β T2 cells by activin is well established (51,52), surprisingly activin down-regulated α GSU expression. It might be that activin regulates α GSU differently in primary and transformed L β T2 gonadotropes, or it could be that proper regulation of α GSU subunit requires paracrine factors provided by other pituitary cell types. Finally, 5Z-7-Oxozeanol fully inhibited oFSH β Luc expression in L β T2 cells cotransfected with the constitutively active activin receptor (Fig. 5B). All of these data showed that TAK1 was necessary for activin-mediated induction of the FSH β -subunit in both the sheep and mouse in transformed L β T2 gonadotropes.

Because oFSH β Luc was induced relatively slowly by activin (after 8 h of treatment), the time-course for TAK1 phosphorylation was investigated and found to be compatible with induction of both p3TPLuc and oFSH β Luc. That is, TAK1 was abundant in L β T2 cells, and its phosphorylation was strongest from 2–8 h but continued to be elevated through 24 h. TAB1 was abundant in L β T2 cells and, presumably, TAB2 and/or TAB3 were also. Further study is needed on TAK1 activation and its relationships to its binding proteins to determine how it brings specificity of action to activin-mediated events.

Finally, TAK1 is reported to activate p38 MAPK in other signaling pathways (64). Therefore, inhibitors of ERK, JNK, and p38 MAPKs were used to determine whether any of the major MAPKs were involved in activin-mediated induction of oFSH β Luc. As expected, the inhibitor of p38 MAPK linked it with activin-mediated induction, but none of the other MAPKs were associated with activin action. These data do not prove that TAK1 is responsible for p38 activation, but the results are consistent with TAK1 activation of p38, which then activates transcription factors that directly or indirectly induce expression of the FSH β -subunit.

In summary, it has been shown that activin stimulates induction of FSH β through a Smad-independent pathway initiated by the activation of TAK1. Furthermore, p38 MAPK, known to be a downstream target of TAK1, was shown to be involved in activin induction of oFSH β -gene.

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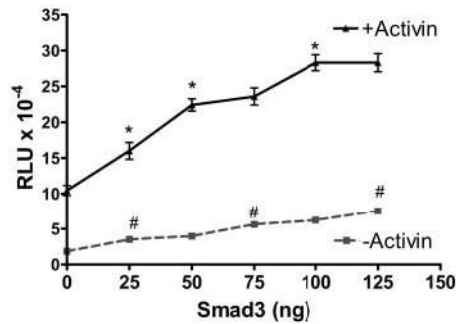
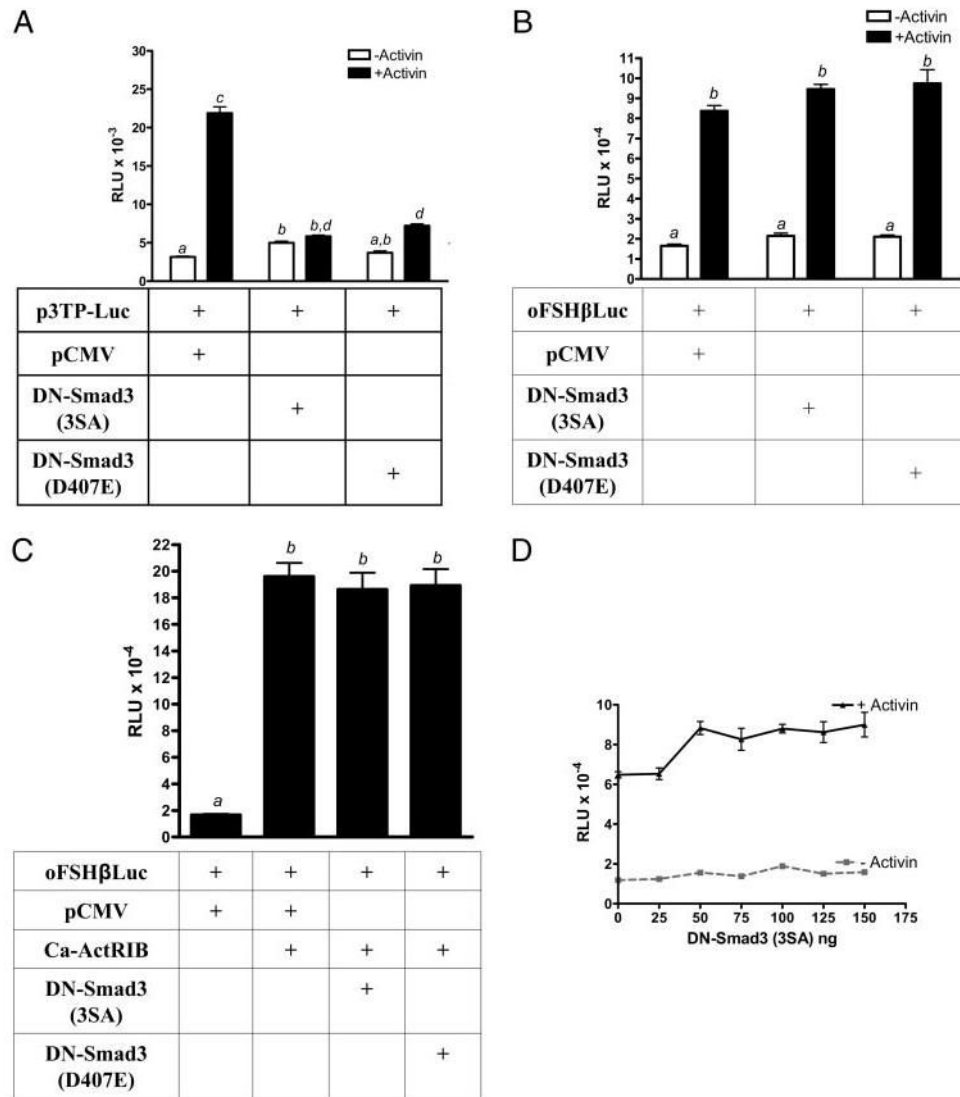


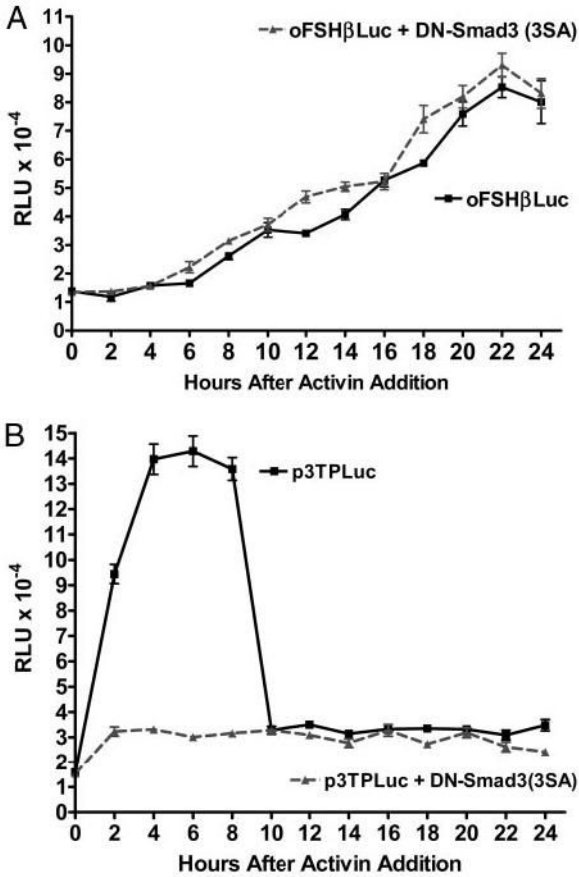
Fig. 1.

Overexpression of Smad3 equally stimulated basal and activin-induced expression of oFSH β Luc. L β T2 cells were plated at 25,000 cells/well in 96-well tissue culture plates. After 24 h, they were cotransfected with 50 ng oFSH β Luc and increasing amounts of Smad3 expression construct (25, 50, 75, 100, and 125 ng). DNA amounts were kept constant at 200 ng DNA transfected per well using pCMV (mock plasmid) to balance amounts of DNA. After transfection (24 h), cells were treated with or without activin (100 ng/ml) for an additional 24 h and assayed for luciferase activity. Ratios of induced/basal were not significantly different from the 5.7 ± 1.6 ratio observed in control cultures not transfected with Smad3 ($P > 0.05$). One-way ANOVA/Tukey's was used to show that increasing levels of Smad3 increased luciferase expression; a significant increase between points ($P < 0.05$) was designated: #, for basal expression; *, for activin-stimulated expression. RLU, Relative light units.

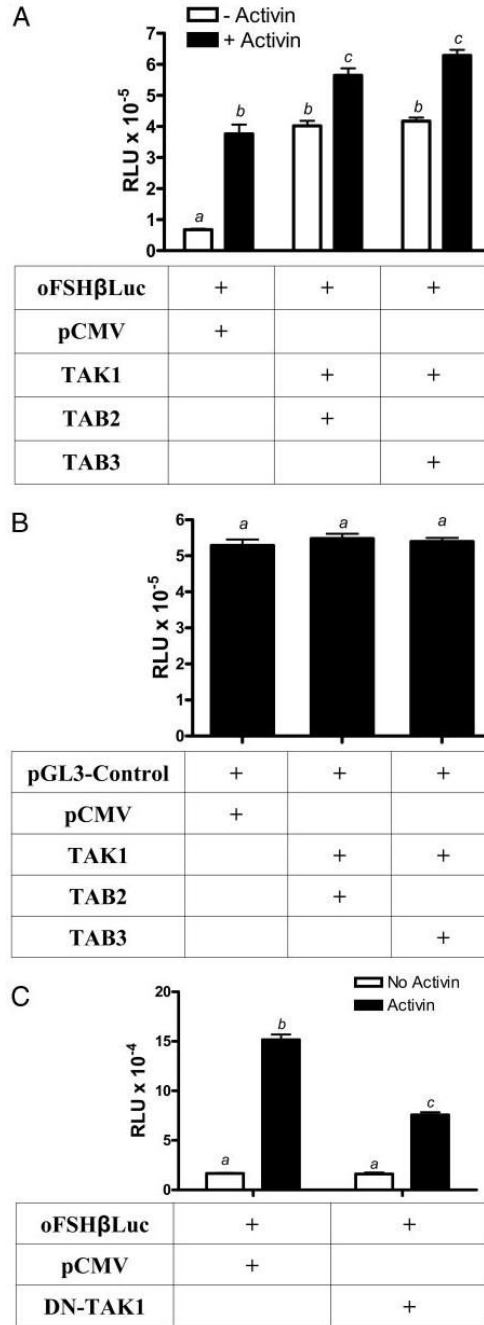
**Fig. 2.**

Activin did not require activated Smad3 to induce oFSH β Luc expression. L β T2 cells were prepared and plated as in Fig. 1 and then treated as follows: A, Cells were cotransfected with 50 ng p3TPLux plus 50 ng pCMV, DN-Smad3 (3SA), or DN-Smad3 (D407E). After transfection (24 h), cells were treated with or without activin (100 ng/ml) for an additional 6 h and assayed for luciferase activity. Means with different letters are significantly different ($P < 0.05$) ($P < 0.001$ for c vs. d). B, Cells were treated as in A except that oFSH β Luc was used instead of p3TPLux. Means with different letters are significantly different ($P < 0.05$). C, Cells were transfected with 50 ng oFSH β Luc plus 100 ng pCMV (column 1) or 50 ng pCMV and 50 ng Ca-ActRIB (column 2). Columns 3 and 4 were transfected with 50 ng of either DN-Smad3 (SA) or DN-Smad3 (D407E) in place of pCMV. Cells were collected 24 h after transfection and assayed for luciferase activity. Means with different letters are significantly different ($P < 0.05$). D, Cells were cotransfected with 50 ng oFSH β Luc and increasing amounts of DN-Smad3 (3SA) expression construct (25, 50, 75, 100, 125, and 150 ng). Total DNA amounts were kept constant using mock plasmid pCMV. Twenty-four hours after transfection, cells were treated with or without activin (100 ng/ml) for an additional 24 h. Ratios of induced/

basal were not significantly different from the 5.5 ± 0.22 ratio observed in control cultures not transfected with DN-Smad3 (3SA) ($P > 0.05$).

**Fig. 3.**

Different kinetics for oFSH β Luc and p3TPLux induction by activin. L β T2 cells were prepared and plated as in Fig. 1. A, Cells were cotransfected with 50 ng oFSH β Luc plus either 50 ng pCMV (•) or 50 ng Smad3 (3SA) (▲). After transfection (24 h), triplicate cultures were sequentially treated with activin (100 ng/ml) every 2 h for 24 h. There was no significant increase in expression for the first 6 h of activin treatment for either control or DN-Smad3 (3SA)-transfected cells. Cells treated with activin for 8 h or longer showed significant increases in expression, but there was no significant difference at any time point between cultures transfected with pCMV (control) or DN-Smad3 (3SA). B, Cells were treated as in A, except p3TPLux was used instead of oFSH β Luc. All cultures treated with activin showed, at least, a significant doubling of expression at all time points ($P < 0.05$). Expression increased further ($P < 0.001$), up to 9.3-fold, between 2 and 8 h, in the absence of DN-Smad3 (3SA), but DN-Smad3 totally blocked this increase.

**Fig. 4.**

TAB2 or TAB3 partner with TAK1 to induce expression of oFSH β Luc like activin. L β T2 cells were prepared and plated as in Fig. 1. A, Cells were transiently cotransfected with 50 ng oFSH β Luc and 150 ng pCMV as mock DNA, or oFSH β Luc and 25 ng TAK1, and 125 ng of either TAB2 or TAB3. Twenty-four hours after transfection, cells were treated with or without activin (100 ng/ml), for an additional 24 h before analysis. Means with different letters are significantly different ($P < 0.05$). B, Cells were transiently cotransfected with 50 ng pGL3-control and either 150 ng mock plasmid pCMV or 25 ng TAK1 plus 125 ng TAB2 or TAB3. After transfection (24 h), cells were assayed for luciferase activity. The results of these treatments were not significantly different ($P > 0.05$). C, Cells were transiently cotransfected

with 50 ng oFSH β Luc and 50 ng of either mock plasmid pCMV or expression vector encoding the TAK1 DN (DN-TAK1-KN). After 24 h of transfection, cells were treated with or without activin (100 ng/ml) for an additional 24 h. *Means with different letters* are significantly different ($P < 0.01$).

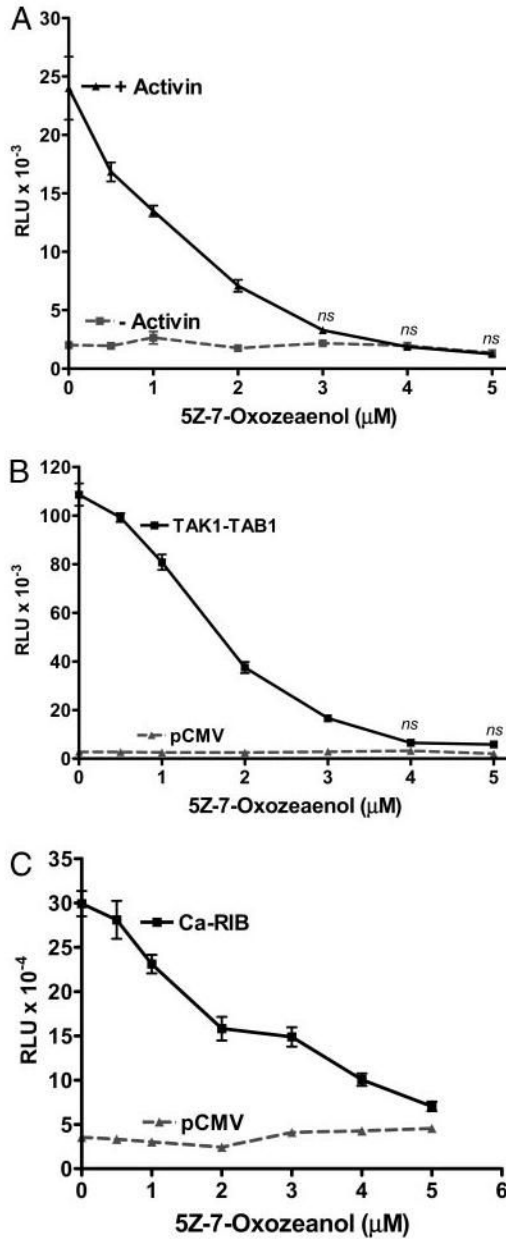


Fig. 5. Inhibition of TAK1 with 5Z-7-Oxozeanol fully blocked activin induction of oFSH β Luc expression. L β T2 cells were prepared and plated as in Fig. 1. A, Cells were transiently transfected with 50 ng oFSH β Luc promoter construct for 24 h. Cells were then treated with or without 5Z-7-Oxozeanol at the indicated concentrations for 2 h before treatment with (\blacktriangle) or without activin (\blacktriangleright) at 100 ng. Cells were incubated another 24 h before analysis for luciferase activity. Because 5Z-7-Oxozeanol is reported to be somewhat labile, halfway through the 24-h incubation, fresh media was added containing the same concentrations of 5Z-7-Oxozeanol and activin. Treatment with 5Z-7-Oxozeanol significantly inhibited induction of oFSH β Luc at all concentrations ($P < 0.05$) and completely inhibited it between 3–5 μM (ns, not significantly different from unstimulated control cultures). B, Cells were cotransfected with 50 ng oFSH β Luc and 50 ng pCMV mock DNA (\blacktriangle) or 50 ng oFSH β Luc, 25 ng TAK1, and 25

ng TAB1 expression constructs (•). After transfection (24 h), cells were treated with increasing concentrations of 5Z-7-Oxozeanol as in A above. Treatment with 5Z-7-Oxozeanol significantly inhibited induction of oFSH β Luc at all concentrations ($P < 0.05$) and completely inhibited it at 4 μ M and above (ns). C, Cells were transiently cotransfected with 50 ng oFSH β Luc and 50 ng of either mock plasmid pCMV (▲) or expression vector encoding the Ca-ActRIB (•). After transfection (24 h), cells were treated with increasing concentrations of 5Z-7-Oxozeanol as in A above. Treatment with 5Z-7-Oxozeanol significantly inhibited induction of oFSH β Luc at all concentrations ($P < 0.05$).

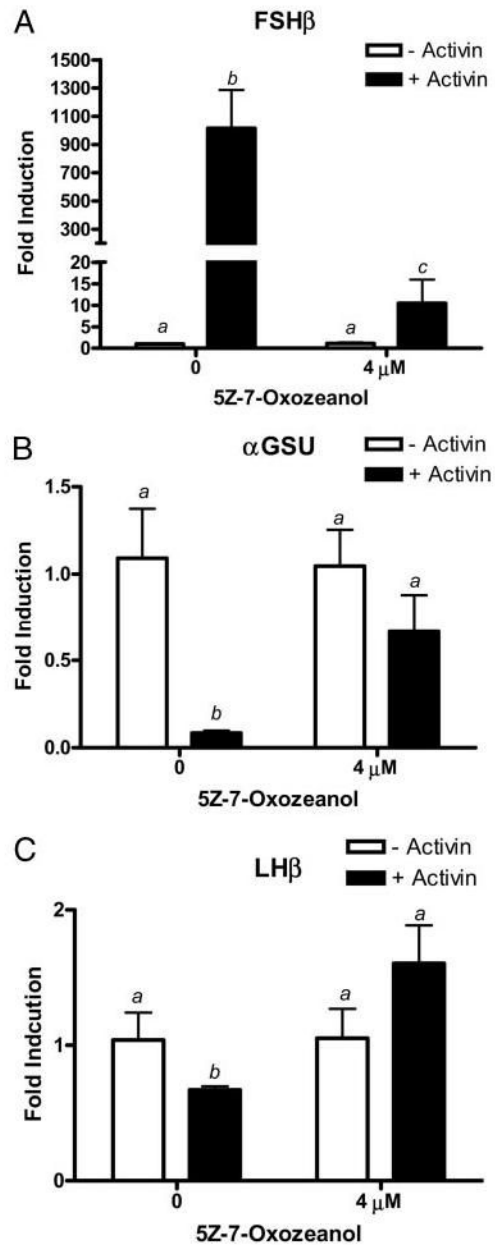


Fig. 6.

LβT2 cells were plated in 6-well plates at 1 million cells per well. The cells were treated with or without activin (100 ng/ml) and treated with or without 4 μM 5Z-7-Oxozeanol. Cells were incubated for 24 h and then washed with PBS, and total RNA was extracted using Tri-Zol reagent. Threshold cycle (Ct) values for (A) FSHβ, (B) αGSU, and (C) LHβ were normalized by subtracting their respective 18s rRNA Ct values. Neither activin nor 5Z-7-Oxozeanol altered 18s rRNA levels because Ct values for 18s rRNA did not change significantly ($P > 0.05$). Normalized Ct values were averaged and used to compare FSHβ, αGSU, and LHβ in the absence or presence of activin using the $2^{-(\Delta\Delta Ct)}$ method for quantitation. The data are plotted as fold-induction of mRNA levels above basal expression. Means with different letters are significantly different ($P < 0.05$).

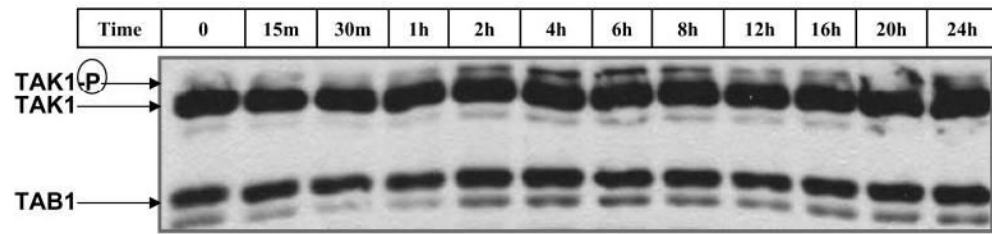
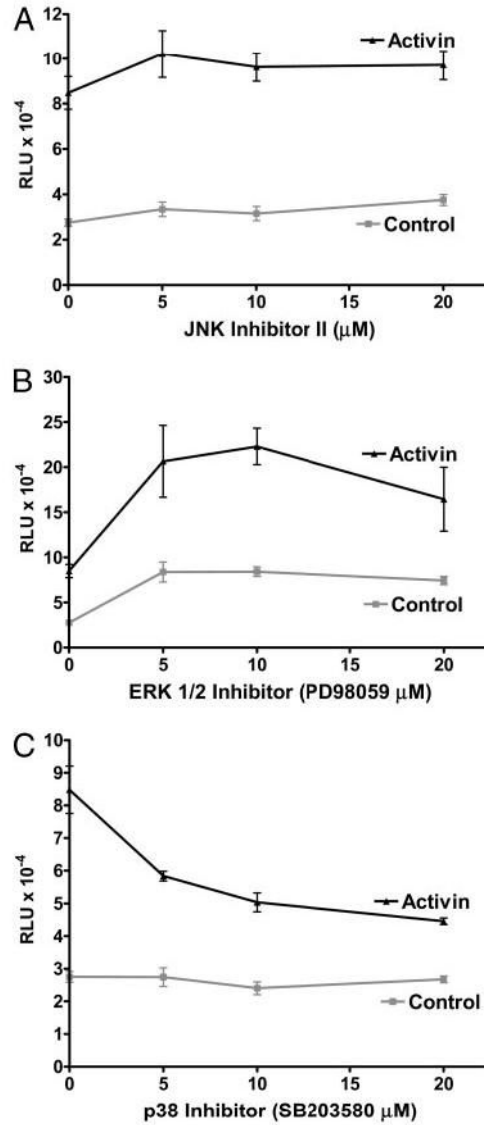


Fig. 7.

Activin phosphorylated TAK1 within 2 h and maintained TAK1 activation for 24 h. L β T2 cells were plated at 1 million cells per well in 6-well plates. Cells were pretreated with follistatin-288 (250 ng/ml; 16 h) and washed with culture media. Cells were then treated with activin (100 ng/ml) for 0, 15, or 30 min or 1, 2, 4, 6, 8, 12, 16, 20, or 24 h. Phosphorylation of endogenous TAK1 was detected by Western blot analysis as described in *Materials and Methods*.

**Fig. 8.**

Inhibition of p38-MAPK partially blocks activin induction of oFSH β . L β T2 cells were prepared and plated as in Fig. 1A and transiently transfected with 50 ng oFSH β Luc. A, After transfection (24 h), cells were treated with or without activin (100 ng/ml) and with JNK inhibitor II (0, 5, 10, and 20 μM); B, the same as A but using the ERK inhibitor; C, the same as A but using the p38 MAPK inhibitor (0, 5, 10, and 20 μM). Cultures were treated for 24 h with or without inhibitors and then analyzed for luciferase activity. Only SB203580 significantly decreased the ratio of induction/basal expression ($P < 0.05$).