

L β T2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to activin A

K E Graham¹, K D Nusser and M J Low

Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201, USA

¹Division of Endocrinology, Oregon Health Sciences University, Portland, Oregon 97201, USA

(Requests for offprints should be addressed to K E Graham)

Abstract

Secretion of luteinizing hormone in response to gonadotropin releasing hormone (GnRH) has been described in the recently developed L β T2 gonadotroph cell line. We evaluated the expression of follicle stimulating hormone (FSH) β mRNA and secretion of FSH from L β T2 cells in response to GnRH and activin A. L β T2 cells were treated with activin A in doses from 0 to 50 ng/ml, with or without a daily 10 nM GnRH pulse, or

with GnRH alone. FSH secretion was stimulated over 6-fold by concomitant GnRH and activin A in a dose-responsive fashion at 72 h of treatment. FSH β mRNA was detectable by ribonuclease protection assay only in cells treated with activin A with or without GnRH. The demonstration of FSH β gene expression in L β T2 cells further validates these cells as mature, differentiated gonadotrophs and as an important tool for the study of gonadotroph physiology.

Introduction

Studies of multiple aspects of the regulatory pathways of the gonadotropin follicle stimulating hormone (FSH) have been severely hampered by the lack of a highly-differentiated gonadotroph cell line that expresses the FSH β gene and secretes FSH. Similar problems with the luteinizing hormone (LH) gene have been circumvented by expression of this gene in placental cell lines as well as in transgenic animals (Keri, *et al.* 1994, McNeilly, *et al.* 1996). Recently, the L β T2 cell line was derived from a pituitary tumor induced by targeted oncogenesis utilizing promoter sequences from the rat LHB gene linked to the protein-coding sequences of the SV40 T-antigen (Tag) oncogene (Turgeon, *et al.* 1994). These cells were reported to express and secrete LH, but not FSH (Turgeon, *et al.* 1996). We report here inducible expression of FSH β subunit mRNA and secretion of FSH in L β T2 cells.

Materials and Methods

Cell culture

L β T2 cells were generously provided by Dr P Mellon and were grown in 6 well plates to approximately 50% confluence in DMEM containing 10% charcoal-treated fetal calf serum, 20 nM dexamethasone, 0.45% glucose, 50 U/ml penicillin G, 50 ug/ml streptomycin, 0.1 mM nonessential amino acids, and 2 mM L-glutamine. Serum was charcoal-treated to avoid potential inhibitory effects of gonadal steroids. For RNase protection assay, cells were grown in 1:1 DMEM/F12 HAM

without phenol red, with L-glutamine and 15mM HEPES supplemented with 1 uM apo-transferrin, 5 ug/ml insulin, 100 uM putrescine and 60 nM sodium selenite. Drug treatments included recombinant human activin A (National Hormone Pituitary Program (NHPP), NIDDK, Dr A F Parlow) in doses ranging from 0-50 ng/ml and/or one hour pulses of 10 nM gonadotropin releasing hormone (GnRH, Sigma, St Louis, MO, USA) one to three times daily or activin alone for up to three days.

Radioimmunoassay and statistical analysis

Conditioned media from approximately 10⁶ cultured cells were collected daily for FSH determination by RIA utilizing a rat FSH kit (NHPP, NIDDK and Dr A F Parlow), as previously described (Kumar *et al.* 1992). Sensitivity was 4.9 ng/ml. Data from six experiments were analyzed together by two-way ANOVA. One-way ANOVA was used to evaluate drug dose and time responses. Post-hoc analyses were done by the Tukey method.

RNase protection assay

Total RNA from approximately 5x10⁶ cultured cells and individual male pituitary glands was isolated and mouse FSH β mRNA was detected by RNase protection assay. Total RNA was hybridized in solution with 2x10⁵ cpm of a low specific activity (estimated 5x10⁶ cpm/ug) [³²P]-rUTP- labeled anti-sense β -actin riboprobe and 9x10⁵ cpm of a high specific activity (est. 2x10⁹ cpm/ug) mFSH β -specific riboprobe

(corresponding to nucleotides 2790-3115 in exon 3 of mFSH β (Kumar *et al.* 1995a)). Unprotected fragments were digested with a mixture of RNase A and T1 (Ambion, Austin, TX, USA). Protected bands were separated on a 5% denaturing polyacrylamide gel and imaged using a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA, USA).

Results

As previously reported by Turgeon *et al.* 1996, under basal conditions, FSH secretion from L β T2 cells was minimal and did not change over 72 h in culture (data not shown). Similarly, cells treated with a single (Fig. 1a) or thrice daily (not shown) one hour pulse of 10 nM GnRH alone also did not demonstrate significant FSH secretion. In contrast, activin A treatment of cells dramatically increased FSH secretion starting at 48 h of treatment (Fig. 1b). The response to activin A was potentiated at earlier time points (24 and 48 h) by simultaneous treatment with a once daily pulse of GnRH, but the maximal response

was unchanged (Fig. 1c). The response to activin A was dose-dependent with significant increases in FSH secretion seen at 48 h starting at doses of 25 ng/ml (Fig. 1d, shown is treatment with activin A at various doses with a concurrent once daily pulse of GnRH; dose responsiveness was also seen with activin A treatment alone, data not shown).

Mouse FSH β mRNA was undetectable in cells under basal conditions nor with GnRH stimulation. However, similar to the FSH secretory response, treatment with various doses of activin A with or without a daily one hour 10 nM GnRH pulse induced mFSH β mRNA detected by RPA (Fig. 2).

Discussion

This is the first report of an established gonadotroph cell line that expresses and secretes both FSH and LH. In the previous report of LH secretion from L β T2 cells (Turgeon *et al.* 1996), FSH β expression was not detected, however this may have been due to the fact that only GnRH and steroid treatments

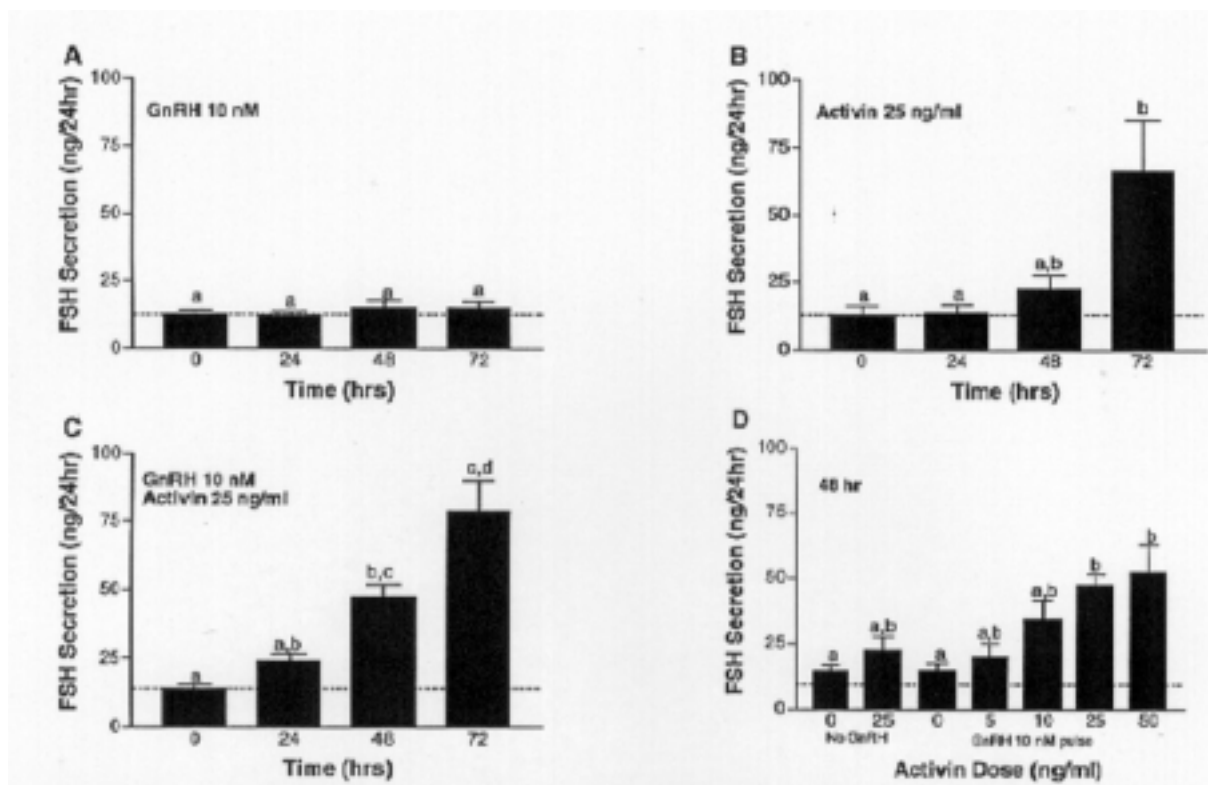


Figure 1 FSH secretion from L β T2 cells in response to GnRH and activin A. For each panel, treatments that differ significantly by post-hoc analysis have no letters in common. Assay sensitivity is denoted by the dotted line. FSH secretion is minimal from untreated cells (not shown) or a) cells treated with a one hour daily pulse of 10 nM GnRH. b) Treatment with 25 ng/ml activin A stimulates FSH secretion 1.7-fold at 48 h and 5.0-fold at 72 h ($P=0.02$). c) Concurrent treatment with 25 ng/ml activin A and one hour daily pulse of GnRH potentiates FSH secretion, with a 3.6-fold increase by 48 h and 6.0-fold increase by 72 h ($P<0.01$). Maximal FSH response to activin A is not changed by addition of GnRH. d) FSH secretion at 48 h in response to increasing doses of activin A with a daily pulse of GnRH ($P<0.005$).

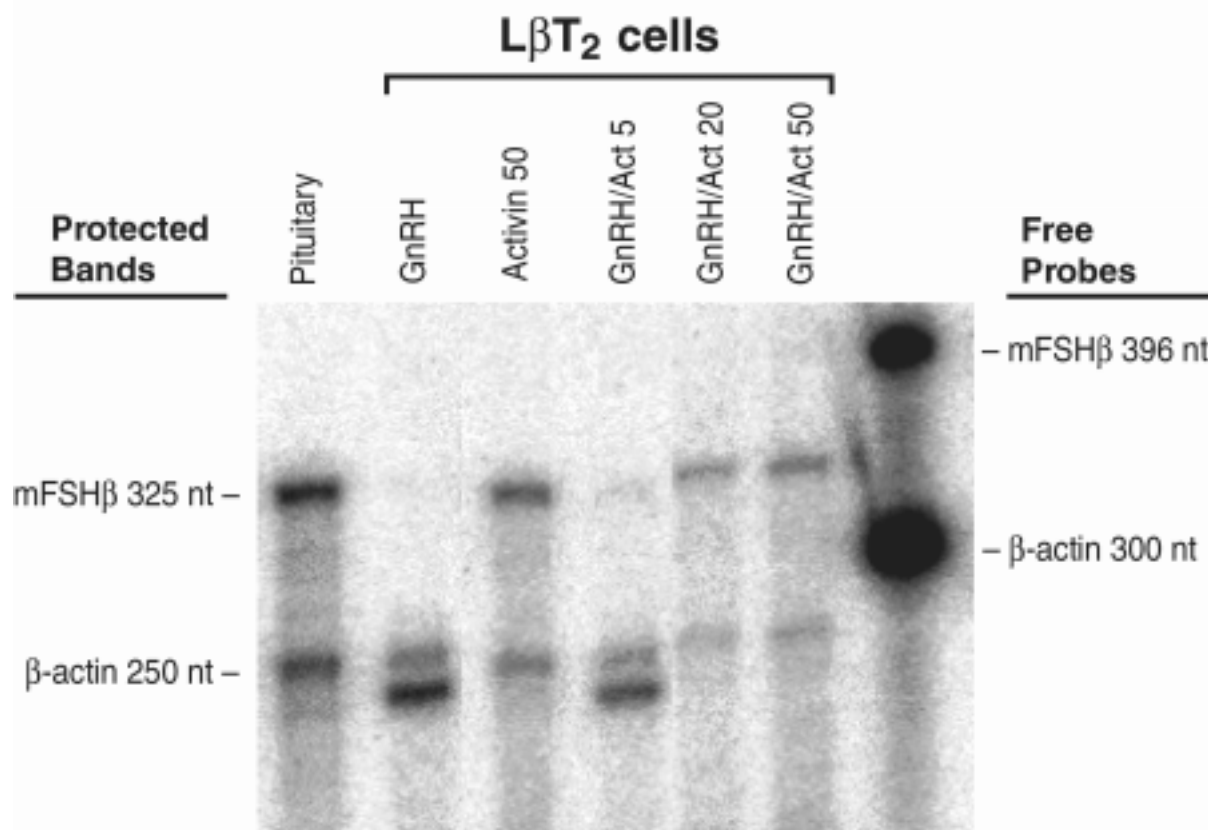


Figure 2 RNase protection assay demonstrating expression of mouse FSH β mRNA in L β T₂ cells treated with GnRH alone, activin alone, increasing doses of activin A (5-50 ng/ml) and a daily one hour pulse of GnRH, all for 48 h. The bands shown are full length mFSH β free probe (396 nt), full length β -actin free probe (300 nt) in the last two lanes, and protected mFSH β (325 nt) and β -actin fragments (250 nt) for each of the L β T₂ and control pituitary samples.

were given. The current study is consistent with those previous findings in that activin was required for stimulation of FSH β expression and FSH secretion. Basal FSH secretion was low and the response to a single daily pulse of GnRH was also minimal. However, in the presence of activin A, FSH secretion increased dramatically on successive days, with synergism observed between the stimulatory effects of GnRH and activin A. The potentiation of FSH secretion on successive days of treatment was also similar to the previous report demonstrating greater maximal LH on successive days.

A second difference between the design of the previous study and the current one that may have influenced the ability to stimulate FSH secretion was the frequency of GnRH pulses. It is well established that lower pulse frequencies stimulate FSH secretion whereas higher pulse frequencies favor LH secretion and even decrease FSH expression and secretion in primary cultured rat pituitary cells (Ishizaka *et al.* 1992, Kaiser *et al.* 1995). When four pulses of 15 min duration were given every 90 min, LH secretion was stimulated (Turgeon *et al.* 1996); in the current experiments, a one hour daily pulse

stimulated FSH secretion in preference to LH (LH data not shown). The interpulse intervals tested in these two studies differ from those previously optimized in primary rat pituitary cultures. However, it is already known that there are differences between L β T₂ cells and rat pituitary cells with regard to GnRH desensitization (Turgeon *et al.* 1996, Weiss *et al.* 1995), although it is not possible to exclude the possibility that these differences were related to the use of static cultures vs. perfusion or paracrine actions from other pituitary cells in the primary culture. In addition, human pituitary adenomas demonstrate aberrant responses to GnRH agonists (Klibanski *et al.* 1989). The optimal pulse frequency will need to be empirically determined in this tumor cell line for both LH and FSH.

The requirement of exogenous activin A administration for FSH stimulation could be explained by a number of factors. There may be a loss of local activin production or overproduction of follistatin as part of the oncogenic process or due to prolonged cell culture in the absence of GnRH. Although GnRH has been shown to increase activin production (Liu *et*

al. 1996), it was ineffective alone in these cells to stimulate FSH secretion. The dose of activin required for FSH secretion is higher than that observed in previous studies with perfused rat primary pituitary cells (Weiss *et al.* 1993), but similar to that in other studies using a static culture model (Attardi & Miklos 1990, Carroll *et al.* 1989) or in other tumor cells (Fernandez-Vazquez *et al.* 1996). Although activin A has been shown to stimulate the synthesis of GnRH receptors (Braden & Conn 1992), this is unlikely to be a significant factor given the robust LH secretory response to GnRH in the previous study (Turgeon *et al.* 1996) indicating the presence of functional GnRH receptors.

The time course of stimulation of FSH secretion is also similar to that seen in previous studies of activin action. We demonstrated increases in FSH secretion and FSH β mRNA starting as early as 24 h after treatment. Stimulation of GnRH receptor synthesis has been reported to occur over 24 to 36 h (Fernandez-Vazquez *et al.* 1996) and although FSH β and activin receptor (ActRI and ActRIIA) expression are observed as soon as 2 h after activin stimulation (Dalkin *et al.* 1996, Carroll *et al.* 1991), FSH secretion in many systems is not observed until after 14 h or more of treatment (Carroll *et al.* 1989, Schwall *et al.* 1988). We have not yet evaluated earlier time points for changes in steady-state FSH β mRNA, however, secretion clearly increases with continued activin treatment. Alternatively, there is evidence that activin has differentiating effects on pituitary cells (Childs & Unabia 1997) and we cannot exclude the possibility that 24 to 48 h of activin treatment induced differentiating changes in these immortalized tumor cells that then allowed FSH β mRNA expression.

Until now, study of the gonadotropin β -subunit genes has been done almost exclusively in transgenic mice or in primary rat pituitary cultures due to the lack of a well-differentiated gonadotroph cell line. Transgenic expression has the advantage of being 'truer' to expression of the endogenous gene, due to the stringency related to chromatin structure and issues such as imprinting, and is particularly advantageous in evaluating the intact hypothalamic-pituitary-gonadal axis allowing study of short and long feedback loops. Primary rat pituitary cultures have been a significant tool, but are not sustainable in culture. Further, although primary rat pituitary cultures in general demonstrate similar regulation to that observed in physiological studies in humans, we have observed some differences among the human FSH β and mouse FSH β genes in transgenic animals and the rat FSH β gene, specifically with regard to androgen regulation (Kumar & Low 1995b). We postulate that the mouse gene may be more comparable to the human gene, making the L β T2 cell line, derived from a mouse pituitary adenoma, a more appropriate model. In addition, we are currently evaluating this cell line for its potential in expressing transfected human FSH β constructs.

The demonstration of expression of FSH β mRNA and FSH secretion further validate the L β T2 cell line as a model of mature, fully differentiated gonadotrophs. A more detailed analysis of the response of L β T2 cells to gonadal steroids and

peptides and GnRH is underway and will further clarify their usefulness for study of gonadotroph physiology.

Acknowledgements

We thank Dr P Mellon for providing the L β T2 cells, Dr A F Parlow and the NHPP for the gracious donation of reagents for the FSH RIA kit and activin, and Ms Christine Fenner for assistance with the manuscript.

References

- Attardi B & Miklos J 1990 Rapid stimulatory effect of activin-A on messenger RNA encoding the follicle-stimulating hormone- β subunit in rat pituitary cell cultures. *Molecular Endocrinology* **4** 721-726.
- Braden TD & Conn PM 1992 Activin-A stimulates the synthesis of gonadotropin-releasing hormone receptors. *Endocrinology* **130** 2101-2105.
- Carroll RS, Corrigan AZ, Gharib SD, Vale W & Chin WW 1989 Inhibin, activin and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Molecular Endocrinology* **3** 1969-1976.
- Carroll RS, Kowash PM, Lofgren JA, Schwall RH & Chin WW 1991 *In vivo* regulation of FSH synthesis by inhibin and activin. *Endocrinology* **129** 3299-3304.
- Childs GV & Unabia G 1997 Cytochemical studies of the effects of activin on gonadotropin-releasing hormone (GnRH) binding by pituitary gonadotrope and growth hormone cells. *Journal of Histochemistry and Cytochemistry* **45** 1603-1610.
- Dalkin AC, Haisenleder DJ, Yasin M, Gilrain JT & Marshall JC 1996 Pituitary activin receptor subtypes and follistatin gene expression in female rats: differential regulation by activin and follistatin. *Endocrinology* **137** 548-554.
- Fernández-Vázquez G, Kaiser UB, Albarracín CT & Chin WW 1996 Transcriptional activation of the gonadotropin-releasing hormone receptor gene by Activin A. *Molecular Endocrinology* **10** 356-366.
- Ishizaka K, Kitahara S, Oshima H, Troen P, Attardi B & Winters SJ 1992 Effect of gonadotropin-releasing hormone pulse frequency on gonadotropin secretion and subunit messenger ribonucleic acids in perfused pituitary cells. *Endocrinology* **130** 1467-1474.
- Kaiser UB, Sabbagh E, Katzenellenbogen RA, Conn PM & Chin WW 1995 A mechanism for the differential regulation of gonadotropin subunit gene expression by gonadotropin-releasing hormone. *Proceedings of the National Academy of Sciences of the USA* **92** 12280-12284.
- Keri RA, Wolfe MW, Saunders TL, Anderson I, Kendall SK, Wagner T, Yeung J, Gorski J, Nett TM, Camper SA & Nilson JH 1994 The proximal promoter of the bovine luteinizing hormone β -subunit gene confers gonadotrope-specific expression and regulation by gonadotropin-releasing hormone, testosterone, and 17 β -estradiol in transgenic mice. *Molecular Endocrinology* **8** 1807-1816.
- Klibanski A, Jameson J & Biller B 1989 Gonadotropin and alpha-subunit responses to chronic gonadotropin-releasing hormone analog administration in patients with glycoprotein hormone-secreting pituitary tumors. *Journal of Clinical Endocrinology and Metabolism* **68** 81-86.
- Kumar TR, Fairchild-Huntress V & Low MJ 1992 Gonadotrope-specific expression of the human follicle-stimulating hormone β -

- subunit gene in pituitaries of transgenic mice. *Molecular Endocrinology* **6** 81-90.
- Kumar TR, Kelly M, Mortrud M, Low MJ & Matzuk MM 1995a Cloning of the mouse gonadotropin β -subunit-encoding genes. I. Structure of follicle stimulating hormone β -subunit-encoding gene. *Gene* **166** 333-334.
- Kumar TR & Low MJ 1995b Hormonal regulation of human follicle-stimulating hormone- β subunit gene expression: GnRH stimulation and GnRH-independent androgen inhibition. *Neuroendocrinology* **61** 628-637.
- Liu ZH, Shintani Y, Wakatsuki M, Sakamoto Y, Harada K, Zhang CY & Saito S 1996 Regulation of immunoreactive activin A secretion from cultured rat anterior pituitary cells. *Endocrine Journal* **43** 39-44.
- McNeilly JR, Brown P, Mullins J, Clark AJ & McNeilly AS 1996 Characterization of the ovine LH β -subunit gene: The promoter is regulated by GnRH and gonadal steroids in transgenic mice. *Journal of Endocrinology* **151** 481-489.
- Schwall R, Nikolics K, Szony E, Gorman C & Mason A 1988 Recombinant expression and characterization of human activin A. *Molecular Endocrinology* **2** 1237-1242.
- Turgeon JL, Windle JJ, Whyte DB & Mellon PL 1994 GnRH and estrogen regulate secretion of LH from an immortal gonadotropin cell line. Program of the 76th Annual Meeting of The Endocrine Society, Anaheim, CA, 1994, p 646 (Abstract)
- Turgeon JL, Kimura Y & Waring DW & Mellon PL 1996 Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. *Molecular Endocrinology* **10** 439-450.
- Weiss J, Cote C, Jameson JL & Crowley W 1995 Homologous desensitization of gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone secretion *in vitro* occurs within the duration of an endogenous GnRH pulse. *Endocrinology* **136** 138-145.
- Weiss J, Crowley WF, Halvorson LM & Jameson JL 1993 Perfusion of rat pituitary cells with gonadotropin-releasing hormone, activin, and inhibin reveals distinct effects on gonadotropin gene expression and secretion. *Endocrinology* **132** 2307-2311.