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Follistatin has characteristics of a primary response gene in porcine granulosa cells

C.E. Lindsell[1,2], V. Misra[3] & B.D. Murphy[4,5]

[1]Department of Obstetrics and Gynecology, [2]Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA, USA; [3]Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, S7N OW0; [4]Centre de recherche en reproduction animale, Université de Montréal, C.P. 5000 St Hyacinthe, PQ, Canada J2S 7C6

To explore the regulation of follistatin gene expression, porcine granulosa cells were incubated with the translational inhibitor, cycloheximide (CHX), for periods from 6–24 h. This resulted in a 3 to 10-fold increase in follistatin mRNA accumulation compared to vehicle treated control cultures. At 20 h, CHX augmented the follicle stimulating hormone (FSH) induced stimulation of follistatin mRNA accumulation by a mean of more than sixfold. Over 6 h, CHX elevated follistatin mRNA abundance twofold, while epidermal growth factor (EGF) increased the message threefold. CHX in the presence of EGF produced an effect additive to the EGF response. Results in the longer term differed, as pretreatment of granulosa cells with CHX for 20 h suppressed the induction of follistatin gene expression by both EGF and phorbol 12-myristate-13-acetate. By blockade of transcription with Actinomyocin D, an estimate of the half-life of follistatin mRNA between 4 and 8 h was made. Half-life did not appear to be affected by the CHX suppression of protein translation. From the observations of the occurrence of follistatin gene expression independent of protein synthesis, superinduction in the presence of CHX and FSH, and the interactions between CHX and EGF, it is concluded that follistatin is a primary response gene in porcine granulosa cells.

Keywords: follistatin; primary response gene; granulosa cells; immediate early gene; epidermal growth factor

Introduction

The ovarian granulosa cell synthesizes a number of peptides, including inhibins, activins and follistatins which influence the secretion of follicle-stimulating hormone (FSH) from the anterior pituitary gland (DePaolo *et al.*, 1991). Follistatin, the most recently isolated of these peptides, is a monomeric glucoprotein that exists in at least six molecular weight forms in follicular fluid (Robertson *et al.*, 1987; Ueno *et al.*, 1987; Sugino *et al.*, 1993). Its variability is due to differential splicing of the messenger RNA, post-translational cleavage of the C-terminal region and variations in glycosylation (Esch *et al.*, 1987; Shimasaki *et al.*, 1988a,b, 1989; Inouye *et al.*, 1991; Sugino *et al.*, 1993). To date the principal, if not sole action of follistatin is to serve as a high affinity, low capacity binding protein for activin (Nakamura *et al.*, 1990). Besides its putative role as an inhibitor of FSH secretion, follistatin may be involved in granulosa cell function directly at the ovarian level as a luteotropic or atretogenic factor (Xiao *et al.*, 1990). Indeed, some forms show a high affinity for the granulosa cell surface and may be anchored there to modulate activin binding to the cell (Sugino *et al.*, 1993).

The abundance of follistatin mRNA in granulosa cells is associated with granulosa cell maturation and differentiation *in vivo*. For instance, follistatin mRNA appears in rat and pig granulosa cells during follicular development at the time of antrum formation, and persists through ovulation, and is present in the luteal cells of the new corpus luteum (Nakatani *et al.*, 1991; Lindsell *et al.*, 1994). This suggests that the follistatin gene is expressed during granulosa cell proliferation and maturation. Follistatin mRNA accumulation also increases during gonadotropin- or cAMP-induced differentiation of cultured granulosa cells, suggesting that activation of protein kinase A (PKA)-dependent mechanisms results in increased follistatin gene transcription (Michel *et al.*, 1992; Lindsell *et al.*, 1994). Tuuri *et al.* (1994) recently reported that human chorionic gonadotrophin (hCG) elevates follistatin mRNA accumulation in the short term in human granulosa-lutein cells in primary culture. In addition, follistatin gene expression in cultured granulosa cells is rapidly induced by EGF and the tumor promoting phorbol ester, phorbol 12-myristate 13-acetate (Lindsell *et al.*, 1993). Stimulation of follistatin mRNA levels by PMA demonstrates that the follistatin gene may be induced by activation of the protein kinase C (PKC) pathway (Lindsell *et al.*, 1993).

Genes that do not require protein synthesis for induction of mRNA synthesis have been termed 'primary response genes' or 'immediate early genes' (Herschman, 1991). These genes are commonly induced by phorbol esters and growth factors. As our previous studies have demonstrated a profound induction of follistatin mRNA accumulation by EGF and PMA, we tested the hypothesis that induction of follistatin gene expression is independent of ongoing protein synthesis in porcine granulosa cells in culture.

Results

Treatment of granulosa cells for 20 h with 40 mM CHX reduced the incorporation of [^{35}S]methionine by 91% ($P<0.01$). A Northern blot which demonstrates the effects of this treatment on steady state levels of follistatin mRNA, a mean increase of more than threefold over control, can be found in Figure 1.

The result of combination of FSH and CHX is also represented in Figure 1. The addition of both CHX and FSH for a period of 20 h resulted in a mean superinduction of the message to approximately twice that observed with FSH alone (Figure 1). The effects of the same dose of CHX over 6 h are presented in Figure 2, which demonstrates a follistatin mRNA response more than twofold greater than EtOH treated controls. As we have previously shown (Lindell *et al.*, 1993), EGF elevated message abundance, in this case by threefold (Figure 2). The effects of concurrent EGF (0.1 and 1.0 nM) and CHX appeared additive, resulting in increases to four to fivefold, approximating the sum of the EGF and CHX responses.

After 20 h of CHX treatment, treatment with EGF for 2 h induced the expected elevations of follistatin mRNA to sevenfold over control values (Figure 3). The 20 h blockade of translation alone more than doubled the accumulation of the follistatin transcript. However, the combination of EGF

Correspondence: B.D. Murphy
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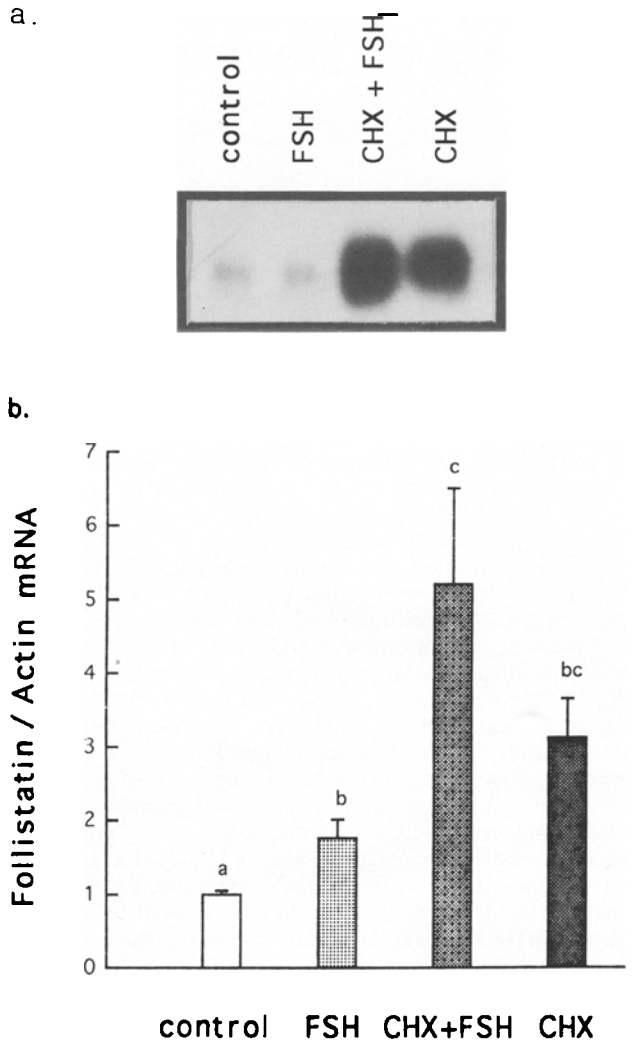

Figure 1 Effect of CHX on the accumulation of follistatin mRNA in porcine granulosa cells. Cells were treated for 20 h with vehicle alone or porcine FSH (100 ng/ml), CHX (40 mM) or FSH + CHX. CHX was added 30 min before FSH. (**a**) represents the autoradiogram of a Northern blot in which the follistatin message migrated at approximately 2.4 Kb. (**b**) is the densitometric evaluation of total cellular RNA by slot blot hybridization (6 μg/slot) with [^{32}P]dCTP-labelled porcine follistatin and human γ-actin cDNA probes. Autoradiographs of slot blots were scanned by laser densitometry and data represent densitometric units for follistatin relative to γ-actin. The mean ± SEM of two separate experiments is presented. Means bearing different superscripts are significantly different at $P<0.05$

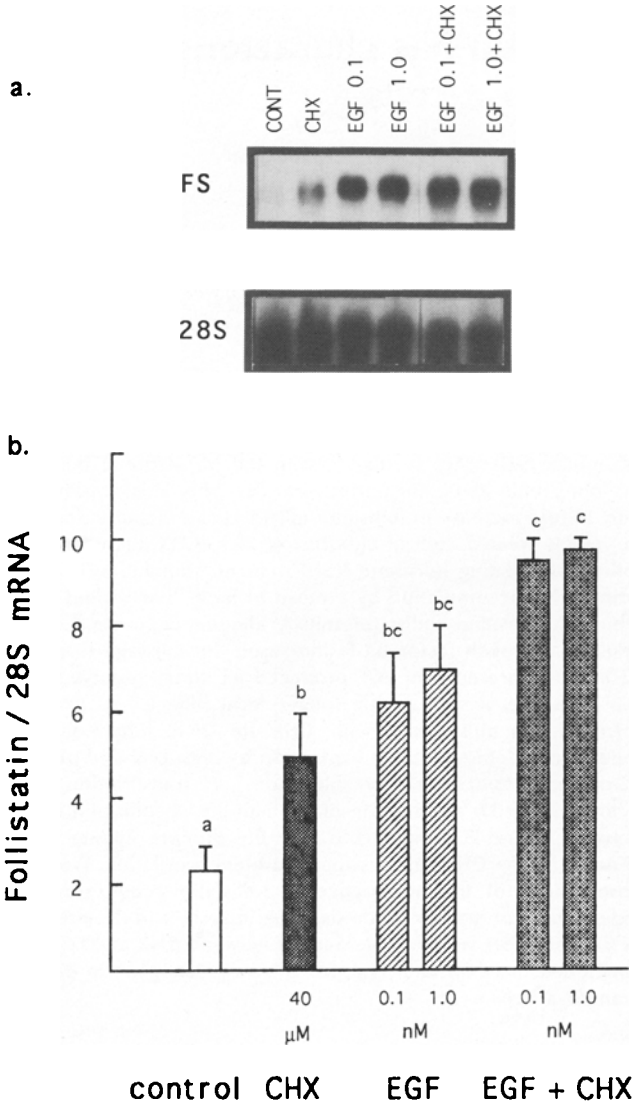

Figure 2 Short term effects of CHX and EGF alone or in combination on the accumulation of follistatin mRNA. Cells were incubated for 6 h with ETOH vehicle (control) 40 mM CHX in EtOH or 0.1 or 1.0 mM EGF in the presence or absence of CHX. (**a**) is the Northern blot of the consequent total RNA which was hybridized first with the [^{32}P]dCTP-labelled porcine follistatin probe then stripped and rehybridized with the 28S probe. Data in (**b**) represent arbitrary densitometric units for follistatin relative to 28S mRNA. The means ± SEM of two separate experiments are presented, and means bearing different superscripts are significantly different at $P<0.05$

and CHX resulted in concentrations of follistatin mRNA which were lower than EGF alone, and not different from CHX alone (Figure 3).

The interactions between suppression of protein translation and phorbol ester-induced follistatin mRNA accumulation were similar to those seen with EGF (Figure 4). CHX treatment alone for 20 h elevated follistatin transcript abundance by sixfold. Treatment of control cultures (i.e. those where there was no CHX pretreatment) with PMA for 2 h resulted in a strong (14-fold) increase in follistatin message. However follistatin mRNA accumulation in CHX pretreated cultures in the presence of PMA was markedly reduced in comparison to PMA alone (Figure 4).

To establish whether the changes associated with CHX treatment could be attributed to alteration of the stability of follistatin transcripts, cell cultures were treated with 5 mg/ml of the transcriptional inhibitor, Actinomyocin D (Act D). The control cultures in these experiments (those that received CHX but not Act D), demonstrated a linear increase in follistatin mRNA accumulation over 24 h (Figure 5). As expected, treatment of cells with Act D alone resulted in a progressive decline in both the steady state and the CHX-induced follistatin mRNA signal over 24 h (Figure 5). Estimates of halflife for the follistatin transcript of between 4 and 8 h were derived from this experiment. Treatment of cultures with CHX altered neither the pattern nor the magnitude of decay in the abundance of follistatin mRNA message (Figure 5).

Discussion

The principal finding of this study is that follistatin mRNA abundance was elevated some 2–10-fold in several experiments in which porcine granulosa cells were incubated from 2–24 h in the presence of the inhibitor of mRNA translation, CHX. The effectiveness of the inhibitor in this system can be discerned from its draconian effects on protein

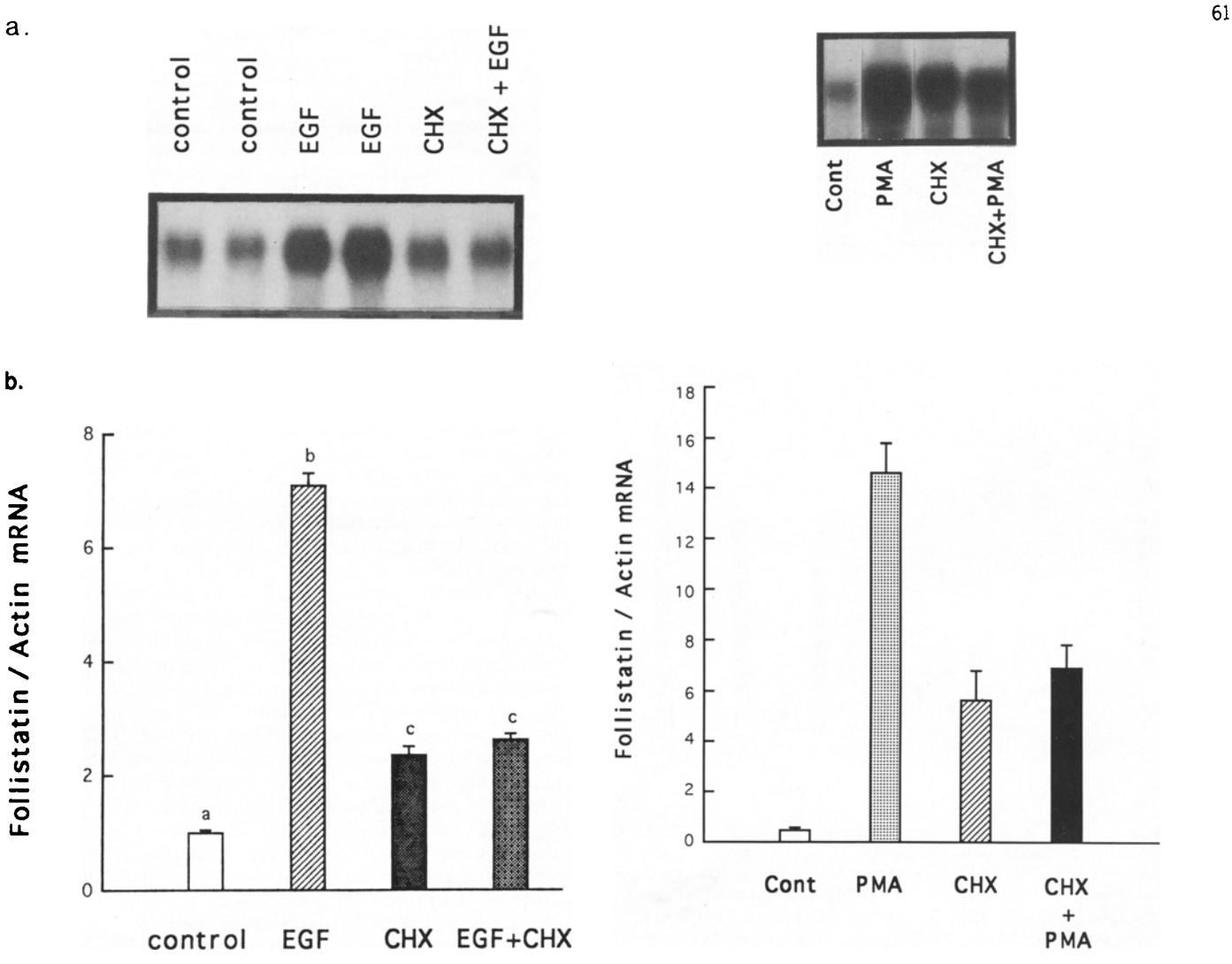

Figure 3 Effect of CHX over the long term on EGF-induced stimulation of follistatin mRNA accumulation in granulosa cells. Cells were treated with CHX (40 mM) or left untreated for 20 h. Untreated cells were incubated alone (control) or with 1 nM EGF (EGF) for 2 h. CHX treated cells were incubated alone (CHX) or in the presence of 1 nM EGF (EGF + CHX) for 2 h. Total cellular RNA was analysed by (**a**) Northern (**b**) slot blot hybridization (6 μg/slot) with [^{32}P]dCTP-labelled follistatin and γ-actin cDNA probes, and autoradiographs were analysed by laser densitometry. Data represent arbitrary densitometric units for follistatin relative to γ-actin mRNA. Bars represent means ± SEM from two separate experiments. Means bearing different superscripts are significantly different ($P < 0.05$)

Figure 4 Effect of CHX on PMA-induced stimulation of follistatin mRNA accumulation in granulosa cells. Cells were pre-treated with CHX (40 mM) or left untreated for 20 h. Cells were then treated with 10 nM PMA for 2 h or left untreated and total cellular RNA was analysed by slot blot hybridization (6 μg/slot) to [^{32}P]dCTP-labelled follistatin and γ-actin cDNA probes, and autoradiographs were scanned by laser densitometry. Data represent means ± SEM of arbitrary densitometric units for duplicate determinations within the same experiment of follistatin relative to γ-actin mRNA

synthesis, resulting in a near total obliteration of [^{35}S]methionine uptake. In addition to superinduction of the follistatin transcript by CHX alone, the present study demonstrates the occurrence of an interaction between EGF and CHX in the short term and FSH and CHX in the longer term. Tuuri *et al.* (1994) have demonstrated a similar superinduction of follistatin message over 24 h in primary cultures of human granulosa cells, by the combination of hCG and CHX. In the human cell model, CHX alone induced only a mild elevation in follistatin message at 2 h (Tuuri *et al.*, 1994). However, this effect increased at 24 h to reach a magnitude similar to that which we report herein.

Primary response or immediate-early genes are characterized by their induction or superinduction in the absence of any intervening protein synthesis, and by their superinduction in the presence of stimulatory ligands (Herschman, 1991). The data presented herein indicate that follistatin fits the definition of a primary response gene in the ovary, based on increases in message abundance in response to CHX and to FSH in combination with CHX, as well as in terms of the responses to CHX and hCG shown by Tuuri *et al.* (1994).

A second defining characteristic of primary response genes is their up-regulation by mitogenic agents (Herschman, 1991). In the present study, in confirmation of our earlier report (Lindsell *et al.*, 1993), we show an early and strong follistatin mRNA response to EGF, which is a potent mitogen to granulosa cells in culture (Gospadarowicz & Bialecki, 1978). The CHX sensitivity of the response to EGF varies in an interesting manner. CHX seems to increase the EGF induced follistatin message accumulation in the short term, but to have an inhibitory effect at 24 h. Thus, as noted below, in the longer term, ongoing protein synthesis is believed necessary for transduction of the EGF signal.

We previously reported that EGF induces follistatin mRNA increases at 2 h followed by a progressive decline between 12 and 24 h, despite the continued presence of the ligand (Lindsell *et al.*, 1993). Therefore, the EGF responses of follistatin meet a third criterion on the definition of primary response gene, their expression is a transient event.

A fourth characteristic of primary response genes is their activation by phorbol esters (Herschman, 1991; Fletcher *et*

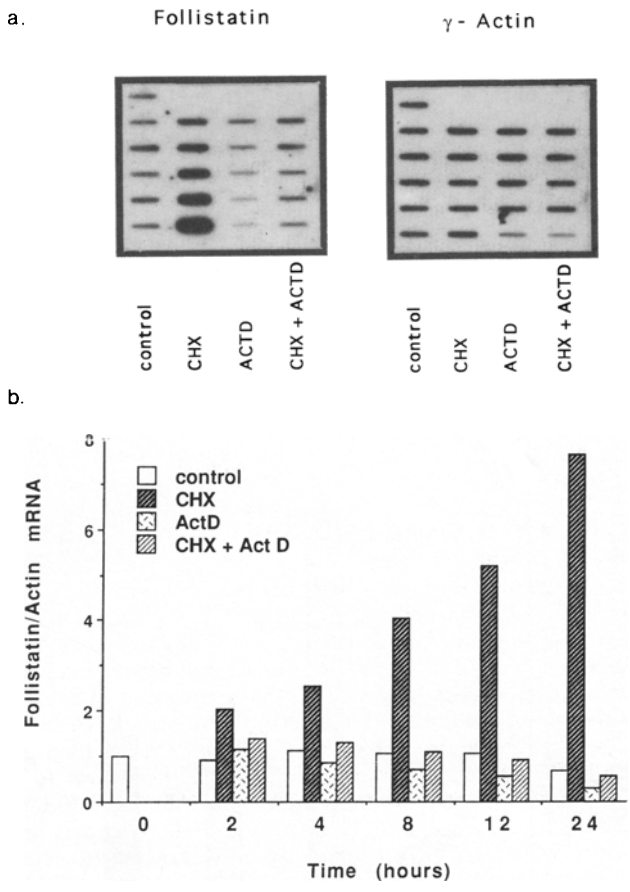

Figure 5 Effect of Act D (5 mg/ml) on accumulation of follistatin mRNA in granulosa cells cultured in the presence or absence of CHX (40 mM). CHX was added 30 min before Act D. Total cellular RNA was harvested 0, 2, 4, 8, 12 and 24 h after addition of Act D, and analysed by slot blot hybridization (6 μg/slot) to [^{32}P]dCTP-labelled porcine follistatin cDNA. Autoradiographs of slot blots (**a**) were scanned by laser densitometry (**b**). Data represent mean arbitrary densitometric units from two separate experiments, normalized to a value of 1.0 for the untreated 0 h cultures

al., 1991). The present demonstration of a remarkable elevation in the levels of the follistatin transcripts in cultures treated with PMA confirms our previous observation (Lindsell *et al.*, 1993) and further defines follistatin as an immediate-early gene. In the present study, long term treatment with CHX resoundingly reduced the PMA response, in symmetry with the long term effects of CHX on the EGF response. Our previous studies of long term down-regulation of the PKC pathway suggest that EGF functions through this intracellular route (Lindsell *et al.*, 1993). This supposition has recently been confirmed by use of the specific PKC inhibitor, Calphostin C (M.L. Fernandez and B.D. Murphy, unpublished results). Thus, we conclude that signal transduction via the PKC pathway to induce expression of the follistatin gene requires protein synthesis.

The results of Act D treatment are interpreted to indicate that the CHX-induced increases in follistatin message abundance are not the result of alteration of the stability of the transcript, as its disappearance after blockade of transcription did not appear to differ in the presence or absence of CHX. It has been hypothesized by Themmen *et al.* (1991) that the effect of CHX on cells in culture is a generalized increase in mRNA stability. We have previously presented evidence for differential responses to CHX in terms of message accumulation in the porcine granulosa cell model in which CHX did not alter the abundance of steroidogenic enzyme messages (Chedrese *et al.*, 1990; Murphy, 1994). CHX did not increase the FSH receptor message, but interdicted its downregulation by FSH (Murphy, 1994). Together, these findings suggest that CHX may act in this model on protein intermediates with a consequent impact on transcription rather than on post-transcriptional events. However, using a similar experimental paradigm, Tuuri *et al.* (1994) concluded that hCG alters follistatin message by altering half-life in human granulosa cells, thus indicating that post-transcriptional regulation of follistatin transcript abundance may occur.

The induction of transcription in early response genes is not well understood. In some cases, there appear to be negative regulatory elements upstream from the coding sequence of the gene in question that inhibit transcription, as with c-*fos* (Lamb *et al.*, 1990). Two such inhibitory regions which suppress basal transcriptional activity have been identified in the rat follistatin gene (Miyanaga & Shimasaki, 1993). This component of the rat follistatin gene bears 92% homology with pig gene and the putative regulatory region of the pig gene contains all of the potential regulatory sequences present in the rat (Shimasaki *et al.*, 1988b). The negative regulatory sequences identified in the rat gene bear little homology to characteristic DNA enhancer/inhibitor regions (Miyanaga & Shimasaki, 1993), thus their function remains obscure. From the present study, the induction of expression of the follistatin gene by blockade of protein translation may indicate that steady regulation of follistatin may involve a labile protein which interacts with these inhibitory regions.

All studies of follistatin gene products to date have concluded that there are multiple pathways of signal transduction and regulation. In the short term, the PKC pathway appears the most important (Lindsell *et al.*, 1993), while over longer periods (12–48 h) the cyclic AMP, PKA-dependent signal transduction may be the principal means of control (Klein *et al.*, 1991; Miyanaga & Shamasaki, 1993; Lindsell *et al.*, 1994; Tuuri *et al.*, 1994). The present investigation extends previous findings to include negative regulation by means of a product dependent on protein translation. The nature of the protein(s) awaits further investigation.

In a recent review, Richards (1994) described immediate-early genes in the mammalian ovary. Several of these including c-*fos* (Delidow *et al.*, 1990) and serum-induceable kinase (Alliston *et al.*, 1994), respond to FSH but not to LH. Follistatin has a similar response pattern (Findlay, 1993; Lindsell *et al.*, 1994), further implicating it as an early response gene. Follicle growth, comprised primarily of granulosa cell mitosis, is stimulated by FSH and EGF in the porcine ovary. Both hormones elevate follistatin in this model, EGF in the short term (Lindsell *et al.*, 1993) and FSH in the longer term (Findlay, 1993; Lindsell *et al.*, 1994).

The ovary and particularly the granulosa cell is quantitatively the most important source of follistatin production (Findlay, 1993). Thus, it may be that the immediate-early responses of follistatin to mitogens are associated with a paracrine role in the ovary. Findlay (1993) hypothesized that follistatin functions in promotion of differentiation of granulosa cells or in warding off the atretogenic effects of activin (Woodruff *et al.*, 1990). This view is consistent with higher levels of follistatin mRNA present in luteinized tissues (Lindsell *et al.*, 1994). However, EGF, which induces granulosa cell mitosis (Gospodarowicz & Bialecki, 1978) and induces the undifferentiated granulosa cell phenotype, causes rapid increases in follistatin expression (Lindsell *et al.*, 1993) presenting an apparent contradiction. Further, it has been recently demonstrated that activin, in combination with FSH, promotes maturation of rat follicles *in vitro* (Li *et al.*, 1995). This effect is reversed by addition of follistatin, suggesting it is playing an inhibitory role. From studies of transgenic ablation of follistatin, Matzuk *et al.* (1995) concluded that the peptide may be modulating other transforming growth factor β related proteins or acting independently. Thus, follistatin may have more than one paracrine function, depending on the state of the differentiation of granulosa cells subject to its effects.

Follistatin was first isolated due to its ability to suppress

FSH secretion (Ueno *et al.*, 1987). It was later shown to function as a high affinity, low capacity binding protein which neutralizes ovarian activin (Nakamura *et al.*, 1990). Recent reports have indicated that follistatin and its effects on activin may have other important functions in vertebrate systems. It is essential during fetal development for postnatal survival in mice (Matzuk *et al.*, 1995). Follistatin blocks activin-induced differentiation of mouse leukemia cells (Shiozaki *et al.*, 1992). Follistatin has been shown to play a direct role in inducing amphibian neural development, by binding activin, (Hemmati-Brivaniou *et al.*, 1994), and it appears to alter capacity of gonadotrophin releasing hormone to induce transcription and secretion of hCG in a choriocarcinoma cell line (Shi *et al.*, 1994). Thus, it would seem that identification of follistatin as a primary response gene has implications beyond the ovary.

Materials and methods

Porcine granulosa cell culture

Granulosa cells were isolated from 3–5 mm diameter follicles from prepubertal pig ovaries, then cultured in MEM (Gibco/BRL, Burlington, ON) containing 0.1 mM MEM non-essential amino acids (Gibco/BRL) 25 mM HEPES 26 mM $NaHCO_3$ (Sigma Chemical Co, St Louis, MO). 0.06 g/l benzylpenicillin, 0.1 g/l streptomycin sulphate, 2.5 mg/ml amphotericin B (Gibco/BRL), and 1 mM NaOH. Cells were cultured at 37°C in 95% humidified air with 5% CO_2 for 48 h in culture medium with 10% heat-inactivated fetal bovine serum (Gibco/BRL), then for 48 h in culture medium without serum. The medium was then changed and treatments were added.

Northern and slot blot hybridization

Total RNA was isolated from granulosa cells as previously described (Kingston, 1989), and analysed by Northern or slot blot hybridization, using a cDNA probe consisting of the entire coding region for porcine follistatin, labelled with [^{32}P]dCTP by random primer extension. Hybridization was carried out as previously described (Lindsell *et al.*, 1993). Briefly, nylon membranes were prehybridized for 2 h at 65°C in hybridization solution (5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 10% dextran sulphate, 200 mg/ml denatured salmon sperm DNA). After prehybridization, membranes were hybridized overnight at 65°C in hybridization solution containing denatured follistatin probe. Nylon membranes were washed twice in 2 × SSPE, 0.1% SDS at room temperature, then twice in 0.1 × SSPE, 0.1% SDS at 65°C, then autoradiographed using Kodak XAR-5 film. The blots were stripped and rehybridized with human γ-actin or 28S RNA probes, as a control for RNA loading and level of constitutive expression. The autoradiograms were scanned by laser densitometry and the results are presented as a ratio of abundance of follistatin to the constitutive probe in arbitrary densitometric units.

Incorporation of [^{35}S]methionine in cultured granulosa cells

Culture medium was removed from granulosa cells after 20 h incubation with CHX or medium alone. It was replaced with methionine-free MEM. After 30 min incubation at 37°C, the medium was replaced with methionine-free MEM containing 70 mCi/ml *L*-[^{35}S]methionine, and cells were incubated for a further 3 h. Medium was aspirated and cells were washed with PBS pH 7.4, then scraped from the plates, applied to a glass filter, lysed with 5% trichloroacetic acid, and rinsed with absolute ethanol. The amount of radioactivity in the labelled cells was determined by liquid scintillation spectrometry.

Experiments

As noted above, all experiments were initiated after 48 h of serum free culture. Each experiment, save that with CHX and PMA (Figure 4), was carried out twice using separate populations of granulosa cells collected from the abattoir on different days. The first experiment comprised the determination of the effects of CHX (40 mM) over 20 h on the abundance of follistatin mRNA, in this and in subsequent trials, two controls were employed, untreated cells and cells that received the EtOH vehicle. In the second experiment, the interactions between long term (20 h) suppression of protein synthesis on FSH induced changes in follistatin mRNA, were studied by concurrent treatment with porcine FSH (Sigma). The third trial was an exploration of the short term (6 h) effects of CHX alone or in combination with EGF (0.1 or 1.0 nM). In subsequent trials the effects of EGF (1 nM) and the phorbol ester PMA, (10 nM) were investigated by determining the responses of cells in terms of follistatin mRNA abundance after incubation of cells for 20 h with CHX (40 mM) or medium alone. In the final experiment, cells were treated with Act D (5 mg/ml) in the presence or absence of CHX, to determine whether the effects of CHX could be explained by an increase in stability and persistence of the follistatin message. Cultures were terminated and total RNA harvested at 0, 2, 4, 8, 12 and 24 h for Northern and slot blot analysis.

Statistical analysis

Homogeneity of variance was first evaluated in each experiment by Bartlett's test, and where heterogenous variance was present, the data were transformed. One way analysis of variance was performed and, in the presence of significant F value, individual comparisons of means were made using the Tukey-Kramer test. Incorporation of [^{35}S]methionine was compared between untreated cells and those treated with CHX and by *t*-test.

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