Regulation of pituitary inhibin/activin subunits and follistatin gene expression by GnRH in female rats

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Abstract

Pituitary inhibin B, activin B, and follistatin are local regulators of FSH. Activin B is a homodimeric molecule (βB–βB), while inhibin B contains an α and a βB subunit. The regulation of gene expression of α, βB, and follistatin by local and endocrine hormones was examined in pituitaries from female rats and in perfused pituitary cells by RT-PCR. Ovariectomy (OVX) induced an elevation in the mRNA level of α and βB subunits and follistatin. Short-term (4 h) treatment of pituitary cells with GnRH decreased both the inhibin and the inhibin/activin βB subunit mRNA levels, while long-term treatment (20 h) with 100 nM GnRH stimulated the expression of both subunits. In contrast, the mRNA level of follistatin was elevated after the short-term GnRH treatment. Long-term exposure of pituitary cells to estradiol and inhibin B suppressed the mRNA expression of βB and had no effect on the expression of α subunit and follistatin. Our results demonstrate that the increased expressions of inhibin/activin subunits and follistatin in the post-OVX period can be induced by the lack of gonadal negative feedback, resulting in a high GnRH environment in the pituitary. This study reports for the first time that GnRH administered in high doses and for a long period stimulates the gene expression of inhibin/activin subunits and thereby may contribute to the stimulatory effect of OVX on the expression of these genes.

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Introduction

Activin, inhibin, and follistatin are key regulators of pituitary FSH production. These factors, together with ovarian steroids and GnRH, define the diverse pattern of LH and FSH secretion. Based on their ability to modulate FSH secretion, inhibins and activins, and follistatin were first identified as FSH-regulating gonadal hormones (Carroll et al. 1989, Weiss et al. 1993). Later, it was discovered that these proteins are also produced in a wide range of tissues, including the pituitary gland (Meunier et al. 1988, Shimasaki et al. 1989).

The structurally related proteins inhibit and activin were originally isolated from ovary and characterized as members of the transforming growth factor β (TGFβ) superfamily (Ling et al. 1985, Miyamoto et al. 1985, Robertson et al. 1985, Vale et al. 1986). Inhibins are dimeric proteins composed of a unique α subunit and one of the two β subunits (A or B), forming inhibin A (αβA) or inhibin B (αβB) (Mason et al. 1985). Activins are homodimer or heterodimer molecules comprised of two β subunits (βA and βB) to produce three isoforms of the mature activin, such as activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (Vale et al. 1986). Activins stimulate FSH synthesis by increasing promoter activity of the FSHβ gene (Huang et al. 2001) and by stabilizing the FSH mRNA (Attardi & Winters 1993). Inhibins counteract the activity of activins in two different ways. First, there is competition for the β subunits during ligand assembly, as both inhibin and activin dimers assemble from a common pool of β subunits (Chapman & Woodruff 2003). Second, inhibins can also bind to the type II activin receptor, without stimulating any of the intracellular pathways but blocking the effect of activin by preventing receptor dimerization (Lebrun & Vale 1997). Surprisingly, the search for the unique inhibin receptor has been unsuccessful; only inhibin co-receptors, such as inhibin-binding protein and betaglycan, could be identified. However, these receptors lack the signaling motif in their intracellular domain (Bernard et al. 2002).

The monomeric glycoprotein follistatin was isolated in the late 1980s (Esch et al. 1987, Robertson et al. 1987). It has two forms generated by alternative splicing (Shimasaki et al. 1988a,b). These isoforms of follistatin derive from transcription of five or six exons (Inouye et al. 1991). Later, a third, intermediate form, which is generated by proteolytic processing of the C-terminus, was also found (Sugino et al. 1993). The action of follistatin is attributed to its ability to bind and biologically inactivate the activins and, at least to some extent, the inhibins also, through the common β subunit (Nakamura et al. 1990, Shimonaka et al. 1991). Follistatin has been detected in various tissues, and its...
expression generally coincides with that of the inhibin/activin subunits (Meunier et al. 1988, Shimasaki et al. 1989). The expression of inhibins, activins, and follistatin in the pituitary gonadotroph and folliculostellate cells indicates that these proteins may act as paracrine/autocrine modulators of FSH production (Bilezikjian et al. 2004). Corrigan et al. demonstrated the paracrine/autocrine effect of activin B by incubation of cultured rat anterior pituitary cells with a MAB specific for activin B. The antibody attenuated the basal secretion of FSH in a concentration- and time-dependent manner, without influencing LH secretion (Corrigan et al. 1991). Unlike activin B, activin A and inhibin A seem to have less of a role in FSH secretion while both the mRNA and protein levels of the \( \beta_A \) subunit are undetectable in pituitary (Meunier et al. 1988, Bilezikjian et al. 1993).

Although several studies focused on this issue, our knowledge on the regulation of the pituitary inhibin–activin–follistatin system is incomplete. It is well established that fast-frequency GnRH pulses stimulate the expression of pituitary follistatin, and that the increased level of follistatin downregulates FSH secretion (Kirk et al. 1994, Besecke et al. 1996). However, the regulation of the inhibin/activin subunit expression is not so clear (Besecke et al. 1996, Bilezikjian et al. 1996, Tebar et al. 2000). Bilezikjian et al. (1996) demonstrated that GnRH inhibits the expression of \( \beta_B \) subunit, while in the study by Tebar et al. (2000), an antagonist of GnRH was shown to suppress the mRNA level of \( \beta_B \). It is known from earlier studies that a regulatory mechanism exists between pituitary inhibin, activin, and follistatin. Activin is able to elevate \( \beta_B \) subunit and follistatin mRNA levels and this effect is inhibited by follistatin and inhibin (Bilezikjian et al. 1996).

To reveal the control mechanisms of the pituitary inhibin–activin–follistatin system, we investigated the effects of endogenous GnRH/gonadotropin overproduction on the mRNA expression of pituitary inhibin/activin subunits and follistatin in ovariectomized (OVX) rats. The changes in serum and pituitary LH and FSH levels after OVX were also detected. In studies in vivo, we examined the specific effects of GnRH, recombinant human (rh) LH and FSH, estradiol (E2), and inhibin B on the gene expression of inhibin/activin subunits and follistatin of perifused pituitary cells. The gene expression of LH and FSH receptors was also investigated.

**Materials and Methods**

**Drugs and chemicals**

rhLH and FSH were obtained from Serono, rh inhibin B from Thermo Scientific, Pierce Biotechnology (Rockford, IL, USA), and 17β-E2 was purchased from Calbiochem (Gibbstown, NJ, USA). GnRH was a gift from János Seprődi (Semmelweis University, Budapest, Hungary). The drugs were diluted in Medium 199 (Sigma–Aldrich).

**Adult and surgery**

Adult, female, 2–3-month-old Wistar-R-Amsterdam rats of 250–300 g body weights were used for all experiments. The rats were housed under controlled conditions (12 h light:12 h darkness schedule at 24 °C) with food and water made available ad libitum. Two weeks before using them for experiments, the rats were tested for estrous cycle by taking daily vaginal smears. Rats showing two consecutive 4-day cycles were used. OVX or sham operation was performed through bilateral lumbar incision under isoflurane anesthesia. One group of OVX rats was killed by decapitation on day 1 (acute OVX) and two groups on day 28 (chronic OVX) after the surgery. To abolish cycle-dependent variations in the target gene expression, the sham-operated control rats were killed in estrous stage. Pituitaries were removed, homogenized, and stored in lysis buffer (NucleoSpin RNA II kit, Macherey-Nagel, Düren, Germany) at −70 °C until RNA was extracted and RT-PCR was performed. Pituitaries and blood samples from the second group of chronic OVX and of sham-operated control rats were used for LH and FSH determination by RIA. Blood samples (0.3 ml) were obtained from the jugular vein of these rats under isoflurane anesthesia before decapitation. LH and FSH were extracted from the pituitary homogenates by 0.1 M HCl and were determined by RIA. LH and FSH concentrations of the pituitaries were expressed as µg/pituitary. All groups consisted of 5–6 rats. This study was approved by the local ethics committee for animal experiments (no: BA02/2000-20/2006).

**Experiments in vitro**

The superfused rat pituitary cell system was carried out as described by Csernus & Schally (1991). Mixed populations of pituitary cells obtained from normal female 2–3-month-old rats showing regular ovarian cycle were used for these experiments. The pituitaries were digested in medium containing 0.5% collagenase (Type 2, Worthington Biochemical Corporation, Lakewood, NJ, USA), dispersed into cell groups, mixed with Sephadex G–10 (Sigma–Aldrich), and put into three chambers of the superfusion system. Each chamber contained pituitary cells from three rats, providing about 2–3×10⁶ cells per channel. Each experiment produced three data (two treated and one control), and the experiments were repeated twice. To obtain steady-state cells the treatments were started after perfusing the cells with medium for 2 h. The cells in chambers 1 and 2 were treated simultaneously with the same drug dissolved in medium for various time periods (40 min, 4, 6, 8, and 20 h), and control cells in chamber 3 were perifused with medium. GnRH was used in concentrations of 10 and 100 nM, LH and FSH were applied at 1 IU/ml, inhibin B at 50 ng/ml, and 17β-E₂ at 100 nM. After stopping the perifusion, subcellular fractions were extracted from the cells by RA1 reagent (Sigma) containing 1% β-mercaptoethanol. The cell extract was separated from
the Sephadex gel by filtering through NucleoSpin Filter units (Macherey-Nagel, Inc., Düren, Germany), and RNA was isolated as described below.

**RNA extraction and isolation**

For total RNA extraction, we used NucleoSpin RNA II kit (Macherey-Nagel). Each pituitary was homogenized in 350 μl lysis solution containing 1% β-mercaptoethanol (Sigma-Aldrich), followed by RNA isolation according to the manufacturer’s protocol. The yield and quality of RNA samples were determined spectrophotometrically at 260 nm, 260/280 and 260/230 nm ratios. The isolated RNA was stored at −70 °C until RT-PCR was performed.

**Semi-quantitative RT-PCR**

Activin/inhibin subunits, follistatin, and GnRH receptor (GnRHR) mRNA levels were assessed using semi-quantitative RT-PCR assays. Total RNA (1 μg) was reverse transcribed to cDNA using the Moloney murine leukemia virus reverse transcriptase enzyme (MMLV-RT, Promega). RT reaction was performed in a final volume of 25 μl containing 4 μM exo-resistant random primer, 0.5 mM dNTP mix (Fermentas, Vilnius, Lithuania), 1-6 units/μl RNasin Rnase inhibitor, 1X MMLV RT buffer, 6-4 units/μl MMLV RT (Promega). RT product (1 μl) was used for each PCR amplification with two primer pairs that would amplify: i) cDNA of β-actin standard gene or ii) cDNAs of inhibin α, inhibin/activin βA, βB or follistatin (for primers sequences, see Table 1). Each reaction contained 0.05 units/μl GoTaq Flexi DNA polymerase, 1X GoTaq enzyme buffer, 1.5 mM MgCl₂ (Promega), 0.2 mM dNTP; and 0.3 μM of each primer. The PCR amplification was conducted with the following cycle profile: 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. Cycle numbers for the different primer pair combinations were determined to terminate the PCR when the amplification of both the β-actin and the target are in the logarithmic phase.

The original copy number of the β-actin mRNA was higher than that of the inhibin/activin subunits or follistatin, and therefore the addition of the standard gene primer pair was always delayed (for cycle numbers, see Table 1). The PCR products were separated on Sybr Safe prestained 2% agarose gels for 13 min (iBase Power System, Invitrogen). All PCR products were detected at the expected molecular weights (Fig. 1). Results were quantified using The Kodak Electrophoresis Documentation and Analysis System (Eastman Kodak Company, New Haven, CT, USA). All data were normalized to the β-actin PCR product and shown in percentage of sham-operated/vehicle-treated controls.

**Detection of LHR and FSHR mRNA**

To detect the expression of LH and FSH receptors (LHCGR and FSHR) in pituitary cells, we used specific primers (LHCGR: 5'-GGC GCC CAT CTC TTT CTT TGC CAT C-3' forward and 5'-GGC TTA CTT GCT CCT GGG AAG CC-3' reverse, 273 bp, FSHR: 5'-GCC GCT CAT CAC TGT GTC CAA GG-3' forward and 5'-GCT CTT TCG GGC ATG GAA GTT GTG G-3' reverse primer, 214 bp) for the amplification of mRNA in untreated perifused pituitary cells and in rat ovary. β-Actin primers are shown in Table 1. A negative control (NEC) sample in which the RT enzyme was absent during RT was prepared from normal pituitary RNA. All PCR products were detected at the expected molecular weights (Fig. 1).

**RIA**

LH and FSH concentrations of the sera and pituitary fractions were determined by RIA using materials obtained from the National Hormone and Pituitary Program (Rockville, MD, USA; Rat LH-11 and FSH-11 antibody, LH-RP-3 and FSH-RP-3 reference preparation, LH-I-9 and FSH-I-9 hormone for iodination). The determinations were performed in duplicates. The sensitivity of the RIA was 0.03 ng for LH

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**Table 1** Primer sequences and PCR cycles used in semi-quantitative PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers</th>
<th>PCR product (bp)</th>
<th>PCR cycles (target gene)</th>
<th>PCR cycles (reference gene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibin α</td>
<td>F-5'- TTC ATT TTC CAC TAC TGC CAT GGT AGC-3'</td>
<td>240</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>R-5'- GAT ACA AGC ACA GTG TTG TGT AAT GA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibin/activin βA</td>
<td>F-5'- AGA GGA CGA CAT TGG CAG GAG-3'</td>
<td>165</td>
<td>36</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>R-5'- AGA GGA CGA CAT TGG CAG GAG-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibin/activin βB</td>
<td>F-5'- AGG CAA CAG TTC TTC ATC GAC TTT CGG-3'</td>
<td>304</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R-5'- AGC CAC ACT CCT CCA CAA TCA TGT T3'-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follistatin</td>
<td>F-5'- CCT ACT GTG TGA CCT GAA ATC T3'-</td>
<td>422</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>R-5'- CTC TCT TTC CTC CTT GCT TCT TCC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin (ref. gene)</td>
<td>F-5'- GTC ACC CAC ACT GTG CCC ATC T3'-</td>
<td>542</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>R-5'- ACA GAG TAC TGG CGC TCA GGA G-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRHR</td>
<td>F-5'- CCG CAA TGG TGG CAT GAA GCC TTC T3'-</td>
<td>192</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R-5'- TAG AGT TCT CAG CCG TGG TCT TGG-3'</td>
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</tbody>
</table>

F, forward primer and R, reverse primer.

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and 0.12 ng for FSH. The inter- and intra-assay variations were 10% or less. LH and FSH concentrations of the pituitaries were expressed as µg/pituitary.

Statistical analysis

Statistical analysis of data was performed by t-test or one-way ANOVA followed by Tukey’s test using the computer software Sigma Stat (Jandel, San Rafael, CA, USA). Differences were considered significant when \( P < 0.05 \).

Results

Effects of OVX on the inhibin \( \alpha \), inhibin/activin \( \beta_A \), \( \beta_B \), and follistatin mRNA levels

The initial PCR standardizing experiments revealed that the pituitary inhibin/activin \( \beta_A \) mRNA level is much lower than the \( \alpha \) or \( \beta_b \) levels, as we had to employ a high number of PCR cycles (36) to detect the PCR product (Table 1). Considering that we had to use 36 cycles to detect \( \beta_A \) but only 26 cycles for \( \beta_B \), the cDNA for \( \beta_A \) needs ten cycles more than the cDNA for \( \beta_B \) to reach the detectable level. Because the copies of DNA increase in exponential manner in the cycles of PCR, they increase to \( 2^{10} = 1024 \) in ten cycles. Thus, the mRNA level of \( \beta_A \) in the pituitary might be about 1000-fold lower than the level of \( \beta_B \). Furthermore, we could not detect any changes in the \( \beta_A \) mRNA level after OVX (data not shown). As this subunit is less significant in local activin/ inhibin assembly and is not regulated by OVX-induced hormonal changes, the expression of \( \beta_A \) subunit was not examined in our further experiments. The levels of inhibin \( \alpha \), inhibin/activin \( \beta_B \), and follistatin mRNA expression were investigated on days 1 and 28 after OVX by semi-quantitative RT-PCR (Fig. 2A). On day 1, no significant changes were found in the expression level of the three genes examined. On day 28 after OVX, the mRNA of \( \alpha \) subunit was increased by 27% \( (P < 0.05) \), the level of \( \beta_B \) by 51% \( (P < 0.001) \), and the expression level of follistatin by 57% \( (P < 0.05) \), compared with sham-operated controls (Fig. 2A).

Effects of OVX on the serum and pituitary LH and FSH levels

Serum and pituitary hormone levels were measured by RIA 28 days after OVX (Fig. 2B). We found a 46-fold increase in serum LH and a 17-fold elevation in serum FSH levels compared with sham-operated rats \( (P < 0.001) \). The concentration of pituitary LH was increased by 16-fold and the concentration of FSH by 3.5-fold \( (P < 0.001; \text{Fig. 2B}) \).

The effect of GnRH on \( \alpha \), \( \beta_B \), follistatin, and GnRH receptor mRNA levels

Perifused pituitary cells were treated with 100 nM GnRH continuously for 40 min or 4, 6, 8, and 20 h, and semi-quantitative PCR was performed to detect changes in \( \alpha \), \( \beta_B \), and follistatin mRNA expressions (Fig. 3A). After the treatment of cells with GnRH for 4 h, a small decrease was observed in mRNA levels of both subunits; a 34% decrease in \( \alpha \) \( (P < 0.05) \) and a 20% decrease in \( \beta_B \) \( (P < 0.01) \). Upon further prolongation of the treatment to 6 h, the inhibitory effect of GnRH gradually attenuated and then disappeared at 8 h of the perfusion. However, when the continuous administration of GnRH was extended to 20 h, a significant elevation could be observed in mRNA levels of both subunits. The level of \( \alpha \) subunit increased by 100% \( (P < 0.001) \) and the level of \( \beta_B \) by 44% \( (P < 0.001) \). In contrast, the mRNA value for follistatin was found to be increased by 34% \( (P < 0.001) \) already after a short treatment of the cells with GnRH for 40 min and peaked at 4 h (65%, \( P < 0.05) \). Then the mRNA level decreased gradually and returned to the control level at 20 h (Fig. 3A). The effect of

Figure 1 Validation of the primers used in our experiments. A 100 bp DNA molecular weight marker is shown ranged from 200 to 700 bp. The bands for inhibin \( \alpha \), inhibin/activin \( \beta_B \), follistatin, GnRHR, \( \beta \)-actin, LHCGR, and FSHR were detected at the expected molecular weights.

Figure 2 (A) The effect of OVX on the mRNA levels of inhibin \( \alpha \), inhibin/activin \( \beta_B \), or follistatin on days 1 and 28 after OVX. The horizontal line symbolizes the mRNA level in sham-operated rats. (B) The effect of OVX on the serum and pituitary LH and FSH concentrations. Error bars represent mean ± S.E.M. of 5–6 rats in all groups. Asterisks indicate significant difference between controls and operated rats \( ***(P < 0.001) \) and \( *(P < 0.05) \).
Figure 3 (A) The effect of continuous treatment of pituitary cells with 100 nM GnRH for various times (40 min, 4, 6, 8, and 20 h) on the mRNA levels of inhibin α, inhibin/activin βB, and follistatin (n=4 for all time points). (B) mRNA levels of pituitary inhibin α, inhibin/activin βB, and follistatin after a continuous perfusion of the cells with 10 nM (n=4) or 100 nM (n=4) GnRH. (C) Representative gel pictures showing the expression of inhibin α, inhibin/activin βB subunits, and follistatin relative to β-actin after a continuous perfusion of the cells with 100 nM GnRH for 20 h. Error bars represent mean ±S.E.M. The horizontal line symbolizes the mRNA level to 37% (P<0.005). 100 nM GnRH was also investigated and was compared with the effect of 100 nM GnRH (Fig. 3B). Continuous perfusion of the cells with 10 nM GnRH for 20 h caused a small increase (34%, P<0.001) in α subunit mRNA level. The mRNA expression of βB and follistatin was not altered by this treatment (Fig. 3B). Furthermore, sustained treatment of pituitary cells with 100 nM GnRH for 20 h had no significant effect on the mRNA expression of the GnRHR.

Figure 4 (A) Effects of long-term treatment of pituitary cells with LH and FSH for 4 and 20 h on the mRNA level of pituitary inhibin α, inhibin/activin βB, and follistatin. Error bars represent mean ±S.E.M. The horizontal line symbolizes the mRNA level in untreated perfused cells. Asterisks indicate significant difference between vehicle- and hormone-treated cells (***P<0.001; **P<0.01; and *P<0.05).

**The effect of LH and FSH on inhibin/activin subunits and follistatin gene expression**

To investigate whether the gonadotropins LH and FSH have a role in the regulation of the pituitary inhibin, activin, and follistatin gene expression and thereby can contribute to the changes in mRNA expression of these genes after OVX, pituitary cells were treated with 1 IU/ml LH or FSH for 4 and 20 h (Fig. 4A). The 1 IU/ml dose of the recombinant enzyme was absent during RT, was prepared from normal pituitary RNA (Fig. 4C). β-Actin primers were used in separate PCR. Equal quantity of β-actin mRNA was found in the cells from rat pituitary and rat ovary. A NEC sample, in which the RT enzyme was absent during RT, was prepared from normal pituitary RNA.

Pituitary cells were treated with either 100 nM E2 or 50 ng/ml inhibin B for 20 h, and mRNA expressions for α, βB, and follistatin were measured by semi-quantitative RT-PCR (Fig. 5A and B). E2 suppressed the mRNA level of βB subunit by 34% (P<0.005) but did not alter the mRNA expression of inhibin-α and follistatin (Fig. 5A). Treatment of the cells with inhibin B for 20 h also reduced the βB mRNA level to 37% (P<0.005; Fig. 5B). The mRNA expressions of α subunit and follistatin were not modified either by inhibin B or by E2 (Fig. 4A and B).

**Discussion**

The pituitary inhibin–activin–follistatin regulatory loop has a major role in the control of FSH production and defines the diverse pattern of FSH and LH secretion (Bilezikjian et al. 2004). Activin and GnRH interplay to stimulate the synthesis and secretion of FSH (Weiss et al. 1993), whereas follistatin...
and inhibin counteract the action of activin by reducing its biological activity and blocking its binding to the receptor (Nakamura et al. 1990, Inouye et al. 1991, Shimonaka et al. 1991). Consequently, the hormones and local factors which regulate the synthesis of pituitary inhibin, activin, and follistatin indirectly influence FSH production.

The aim of this study was to reveal the hormones and local factors which play a role in the regulation of pituitary inhibin/activin/follistatin gene expression and can be accounted for the increased expression of these genes in long-term OVX rats. Although the impact of OVX on the mRNA expression of inhibin/activin subunits and follistatin has been published earlier, the mechanisms mediating the effect of OVX are not identified in full detail. In agreement with previous studies (Dalkin et al. 1998, Prendergast et al. 2004), we found that inhibin α, inhibin/activin βb, and follistatin mRNA levels were increased after OVX. However, the expression of βA subunit was hardly detectable in pituitary and was not changed by OVX in our experiments. Moreover, others have been unable to detect the RNA of the βA subunit with S1 nuclease analysis in rat pituitary (Meunier et al. 1988). The low level of gene expression and the fact that no change in βA expression level could be detected after OVX suggests that the A forms of inhibin/activin have very little or no involvement in the local FSH-regulatory loop.

According to the results of our previous study (Kovacs et al. 2001), we showed a substantial elevation in LH and FSH secretion in long-term OVX rats. In this earlier study, we also provided evidence for the pivotal role of GnRH in the stimulation of gonadotropin secretion after OVX by demonstrating that GnRH receptor antagonist cetorelix entirely prevented the stimulatory effect of OVX on LH secretion (Kovacs et al. 2001). The high GnRH environment of pituitary was also shown in long-term OVX and adrenalectomized rats (Sherwood & Fink, 1980). Despite the indirect inhibitory effect of adrenalectomy on GnRH production through corticotropin-releasing factor (CRF), of which production increases after adrenalectomy, the GnRH concentration in the pituitary portal blood was found to be substantially and constantly elevated in these OVX rats (Li et al. 2010).

Considering that the level of various hormones, such as gonadal steroids and gonadotropins in the circulating blood and GnRH in the pituitary portal blood, changes after OVX, any of these hormones could play a role in the regulation of pituitary inhibin/activin subunits and follistatin gene expression. To reveal the regulatory agents that mediate the effect of OVX in these genes, we investigated the direct effect of the hormones and local factors that change after OVX on the inhibin α, inhibin/activin βb, and follistatin mRNA level of pituitary cells in vitro. Because our in vitro studies were motivated by the results of previous studies in OVX rats (Dalkin et al. 1998, Prendergast et al. 2004), we performed OVX experiments and determined serum gonadotropin levels of the OVX rats to demonstrate the effects of OVX in our experimental conditions. To mimic the high GnRH environment of the pituitary developing after OVX, we used high doses of GnRH in vitro. As 1 nM is known to be a physiological dose of GnRH to release LH from rat gonadotrophs in vitro (Kovacs & Schally 2001, Kovacs et al. 2001), we used 10- and 100-fold of the physiological dose. To investigate the time-dependent effect of the high GnRH environment of pituitary on the gene expression of inhibin/activin and follistatin, we applied high-dose GnRH perfusions of the cells for various times from 20 min to 20 h. It is well known that GnRH stimulates the gene expression of follistatin in pituitary (Kirk et al. 1994). In contrast, the effect of GnRH on the expression of inhibin and activin is not clear. Bilezikjian et al. (1996) found that a short-term (2 h) exposure of pituitary cells to GnRH downregulated the mRNA expression of βb subunit. Similar to this finding, we also showed an acute suppression of α and βb subunit mRNA level by GnRH in our experiments. However, on the basis of these results, it is hard to elucidate how the increased level of activin and inhibin develops in the post-OVX period. We demonstrate in this study that although a short-term GnRH treatment inhibits the gene expression of both α and βb subunits, the prolonged presence of GnRH has a stimulatory effect on their production. These results are the first to show that GnRH has diverse short- and long-term effects on the gene expression of inhibin/activin subunits and thereby clarify the development of increased gene expression of activin and inhibin observed in long-term OVX rats. A gradual upregulation of the GnRH receptor expression in pituitary after OVX may enhance the stimulatory effect of GnRH on gonadotrophs (Kovacs et al. 2001).

The gene expression of follistatin was stimulated by GnRH, except at 20 h, but it was not altered by either estrogen or inhibin in our experiments. This observation supports the earlier finding that the main regulator of follistatin is GnRH (Kirk 1994). The gradual attenuation of

**Figure 5** The effect of continuous treatment of pituitary cells with 100 nM estradiol (E2) for 20 h (A) or 50 ng/ml inhibin for 20 h (B) on the mRNA level of inhibin α, inhibin/activin βb, and follistatin, including representative gel pictures of the relative expression of inhibin βb subunit after the treatments. Error bars represent mean ± S.E.M.; n = 4 for both estradiol and inhibin. The horizontal line represents the mRNA level in untreated perifused cells. Asterisks indicate significant difference between vehicle- and hormone-treated cells (**P<0.001 and *P<0.05).
follistatin gene expression during the long exposure of cells to GnRH can be explained by the temporal changes in GnRH signaling. Earlier studies have shown that long-term continuous administration of GnRH was ineffective for stimulating gonadotropin β subunits, whereas the α subunit level was elevated after a GnRH exposure of the pituitary cells for 24 h (Shupnik & Fallest 1994), indicating the existence of a constant and a pulse-dependent component in GnRH signaling (Vasilev et al. 2002). Accordingly, the expression of follistatin might rely on GnRH pulse frequency, whereas the expression of inhibin/activin subunits can be stimulated by constant GnRH signaling. It has been reported that the cytoplasmic carboxyl tail of the GnRH receptor, which is responsible for the agonist-dependent acute desensitization and internalization, is absent in the mammalian receptor (Willars et al. 1999). Moreover, we detected no change in the receptor mRNA expression after a sustained treatment of pituitary cells with GnRH. However, despite the preserved expression of the receptor, the receptor binding and signaling capacity can change during the long-term exposure of the gonadotrophs to GnRH. Receptor uncoupling and temporal impairment of the signaling pathways were reported to cause desensitization of the LH-releasing capacity of the gonadotrophs to GnRH stimulation (Chang et al. 1988). In contrast, the synthesis of gonadotropin α subunit increases after a sustained GnRH treatment (Shupnik & Fallest 1994). This indicates that although the receptor-coupled intracellular pathways are partly downregulated by the constant activation, a component of the pathway remains functional. Based on these findings, a temporal impairment of the signaling pathways can be accounted for the time-dependent changes in mRNA expressions of follistatin in response to sustained administration of GnRH for 10–20 h in our experiments.

The secretion of gonadotropins is increased in long-term OVX rats (Ramirez & Sawyer 1974), but the local role of LH and FSH in the pituitary is not well established. It is still not known if primary gonadotrophs contain functional LH or FSH receptors. Gonadotroph cell line αT3 was shown to express LH receptors, and the activation of the receptors was able to elevate gonadotropin α subunit mRNA level (Huang et al. 1995). Moreover, Lei et al. (1993) found low mRNA expression of the LH receptor, and we also showed the presence of receptor transcript in pituitary. These findings together with the current observation that long-term treatment of pituitary cells with LH suppressed the expression of βB suggest that LH might be able to act in a paracrine/autocrine manner in pituitary. Similar to pituitary, Liu et al. (2001) demonstrated in human granulosa lutein cells that high levels of gonadotropins inhibited the expression of the βB subunit. In contrast to LH, there is no evidence for the presence of functional FSH receptors in the pituitary gonadotrophs. Although we could detect the presence of the FSH transcript, the treatment of pituitary cells with FSH had no effect on the expression of the three genes tested. The lack of FSH action to influence the mRNA expression of inhibin/activin and/or follistatin in our experiments indicates that only LH but not FSH may play a role in the autocrine/paracrine regulation of these genes. It is possible that the lack of FSH action is due to the lack of functional receptors for FSH on the gonadotrophs. Further experiments are needed to determine the protein expression of FSH receptor and to investigate possible responses of the cells to receptor activation.

It was shown in an earlier study that activin is able to elevate βB mRNA expression, and inhibin is a potent inhibitor of the auto-stimulatory effect of activin (Bilezikjian et al. 1996). The existence of this auto-regulatory mechanism by activin/inhibin is supported by our finding that inhibin B decreases the mRNA expression of βB. The treatment of pituitary cells with E2 also decreased the mRNA level of βB expression in our experiments. This finding may help explain why the replacement of E2 in gonadectomized rats prevented the increase in inhibin/activin βB expression (Dalkin et al. 1998). On the other hand, because GnRH concentration is highly increased in the portal blood after OVX (Sherwood & Fink 1980), and sustained GnRH administration at high dose stimulates the expression of βB, the negative feedback effect of E2 to hypothalamic GnRH might contribute to the direct negative effect of E2 to prevent the increase of βB expression after OVX. In physiological conditions, however, the concentration- and time-dependent reactivity of gonadotrophs is different from that found after OVX, and physiological concentrations of E2 can enhance the sensitivity of gonadotrophs to GnRH (Colin & Jameson 1998). As a result, the stimulation of inhibin/activin expression might occur at lower GnRH concentrations or physiological GnRH pulsation. Further investigation is required to determine whether there is cooperation between GnRH and E2 in the regulation of inhibin/activin subunits expression in gonadotrophs in vivo. Our findings, in agreement with earlier results, show that ovarian hormones, such as inhibin and E2, negatively regulate the gene expression of pituitary inhibin and activin in the absence of GnRH (Bilezikjian et al. 1996, Dalkin et al. 1998). Based on these findings and the results from our in vitro experiments, the stimulatory action of GnRH, together with the lack of ovarian E2 and inhibin, could cause the increase of βB gene expression in long-term OVX rats. As the expression of α subunit was also stimulated by the long-term administration of GnRH but it was not suppressed by E2 or inhibin, the role of GnRH in the stimulation of the inhibin–specific α subunit seems to be more significant than in the stimulation of βB.

In summary, this study demonstrates that the expression of pituitary activin/inhibin subunits is regulated by local and peripheral hormones. E2, inhibin B, LH, and short-term GnRH treatment decrease the mRNA expression of βB subunit, while long-term GnRH treatment stimulates βB expression. GnRH has similar effects on the inhibin α and inhibin/activin βB subunits, whereas E2, inhibin B, and LH have no significant effect on the gene expression of α subunit.
Our results provide the first evidence that GnRH administered in high dose and for a long period directly stimulates the gene expression of pituitary inhibin/activin and thereby may participate in the stimulatory effect of OVX on the expression of these genes.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**References**

Attardi B & Winters SJ 1993 Decay of follicle-stimulating hormone-beta messenger RNA in the presence of transcriptional inhibitors and/or inhibin, activin, or follistatin. *Molecular Endocrinology* 7 668–680. (doi:10.1210/me.7.5.668)

Bernard DJ, Chapman SC & Woodruff TK 2002 Minireview: inhibin binding protein (InhBP/p120), betaglycan, and the continuing search for the inhibin receptor. *Molecular Endocrinology* 16 207–212. (doi:10.1210/me.16.2.207)


Bilezikjian LM, Corrigan AZ, Blount AL & Vale WW 1996 Pituitary follistatin and inhibin subunit messenger ribonucleic acid levels are differentially regulated by local and hormonal factors. *Endocrinology* 137 4277–4284. (doi:10.1210/endo.137.10.4277)


Lebrun JJ & Vale WW 1997 Activin and inhibit in have antagonistic effects on ligand-dependent heteromerization of the type I and type II activin receptors and human erythropoietin differentiation. *Molecular and Cellular Biology* 17 1682–1691.


Ling N, Ying SY, Ueno N, Esch F, Desoroy L & Guillemin R 1985 Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. *PNAS* 82 7217–7221. (doi:10.1073/pnas.82.21.7217)


Tebar M, de Jong FH & Sanchez-Criado JE 2000 Regulation of inhibin/activin subunits and follistatin mRNA expression in the rat pituitary at early estrus. Life Sciences 67 2549–2562. (doi:10.1016/S0024-3205(00)00839-0)


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