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Effects of LHRH, FSH and Activin A on Follistatin Secretion from Cultured Rat Anterior Pituitary Cells

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Abstract. We demonstrated the release of follistatin, an activin-binding protein, from cultured rat anterior pituitary cells by measuring immunoreactive (ir-) follistatin in a specific immunoradiometric assay. Ir-follistatin release gradually increased in cultures over 1–18 days and reached its maximal level at 12–15 days of incubation. The basal ir-follistatin levels in the culture media increased about 3- (P<0.01) and 5-fold (P<0.001) in 2 and 10% fetal calf serum for 6 days, respectively. LHRH and activin A caused an approximately 2.0- (P<0.05) and 1.8-fold (P<0.05) rise in ir-follistatin release, respectively, in contrast to the lack of significant FSH effects. The culture medium condensed on sulfate-cellulofine gel was resolved by polyacrylamide gel electrophoresis and blotted with anti-follistatin polyclonal antibody, resulting in at least three protein bands ranging from 35 to 50 kDa under non-reducing conditions. These results indicated that follistatin is produced in anterior pituitary cells and that its secretion is regulated at least in part by LHRH and activin, implying an autocrine/paracrine role of activin and follistatin in the pituitary.

Key words: Follistatin, Activin, LHRH, FSH, Anterior pituitary cells, Immunoradiometric assay (IRMA) (Endocrine Journal 43: 321-327, 1996)

FOLLISTATIN was initially purified from porcine and bovine follicular fluids based on its ability to suppress pituitary FSH secretion [1]. Although follistatin is a single-chain glycoprotein and structurally different from activin and inhibin, it specifically binds to activin and neutralizes its action [2]. Two forms of follistatin mRNA are generated from a single follistatin gene by alternative splicing and translated into two core proteins consisting of 315 and 288 amino acids (FS-315 and FS-288, respectively) [3]. FS-315 occurs as an unglycosylated core protein of 35 kDa and a glycosylated form of 38 kDa. FS-288 is also expressed as a core protein of 31 kDa and a glycosylated protein of 35 kDa [4]. Studies on follistatins purified from porcine ovaries have revealed the presence of six different molecular weight proteins, all of which exert similar activin-binding activity [5].

The secretion of LH and FSH is regulated by circulating gonadal steroids and hypothalamic LHRH. Gonadal proteins, such as inhibin, activin and follistatin, have been identified in various extraglandal tissues and they exert specific effects on FSH gene expression, secretion and synthesis [6–9]. Follistatin suppresses the secretion and cellular content of FSH without affecting FSH mRNA levels in vitro [9]. Follistatin is present in the pituitary and its expression correlates well with that of activin [10, 11]. Bilezikjian et al. have demonstrated that follistatin immunoreactivity is secreted from rat pituitary cells as a broad band of 35 to 46 kDa protein under non-reducing conditions [12]. These findings suggest that follistatin is important as an autocrine/paracrine regulator of FSH release in the
pituitary, but the factors which regulate follistatin secretion are unknown because of difficulty in measuring follistatin concentrations. In this study, we investigated the effects of LHRH, FSH and activin A on immunoreactive (ir-) follistatin secretion from cultured rat anterior pituitary cells by means of a specific immunoradiometric assay (IRMA) that we developed [13].

Materials and Methods

Reagents

Recombinant human (rh-) follistatin and rh-activin A were provided by Dr. Y. Eto (Ajinomoto Central Research Laboratories, Kawasaki, Japan). This rh-follistatin was generated from a human follistatin gene encoding 315 amino acids (FS-315). Porcine activin B was a gift from Professor H. Sugino (Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan). Bovine inhibin was a gift from Professor Y. Hasegawa (Kitasato University School of Veterinary Medicine and Animal Sciences, Towada, Japan). Rat FSH, LH and GH were provided by NIDDK, USA. Synthetic LHRH was purchased from Tanabe Pharmaceutical Co., Tokyo, Japan. A rabbit antiserum to follistatin was generated in our laboratory by immunizing Japanese male rabbits with rh-follistatin [13].

Pituitary cell culture

For each experiment, 40 female Wistar rats at random cycling were decapitated and the pituitary glands were quickly removed. The anterior pituitary cells were dispersed with collagenase and trypsin as described [14]. Cells were plated at a density of $1 \times 10^6$ cells/ml/well in 24-well plastic tissue culture dishes with HEPES-buffered Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO Laboratories, USA). The cells were culture with fresh DMEM containing 10% fetal calf serum (FCS) for an initial 72 h for the preconditioning for long-term cultures. The cells were then rinsed twice with FCS-free DMEM and each experiment was started. The cells were maintained at 37 °C in 5% CO$_2$ and 95% air with 2% FCS-DMEM in the presence or absence of LHRH, rFSH and rh-activin A, and the culture was continued without the replacement of media for the scheduled period. The culture media were then collected and stored frozen at −20 °C until the determination of ir-follistatin by the IRMA. The number of viable cells during the experiment was estimated by trypan blue dye exclusion, and it was about 90% at the beginning of the experiment.

Immunoradiometric assay for follistatin

Ir-follistatin in the culture medium was measured by means of an IRMA that we developed [13]. Briefly, Star tubes (Nunc, Kamstrup, Denmark) were coated with 200 µl of anti-follistatin mouse monoclonal antibody (4-6D9, 2.5 µg/ml) in 0.1 M sodium bicarbonate (pH 9.6) at 4 °C for 24 h and blocked with 1% Block Ace (Teikokuzoki Pharmaceutical Co., Tokyo, Japan). After washing three times with 0.05 M phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween-20, 100 µl of standard or sample and 100 µl of anti-follistatin rabbit polyclonal IgG (1:1000) diluted with assay buffer (0.05 M PBS, pH 7.4, containing 0.4% Block Ace, 1% normal goat serum, 0.1% Tween-20 and 0.05% sodium azide) were added and incubated at 4 °C for 24 h. After washing three times, 200 µl (100,000 cpm) of $^{125}$I-goat anti-rabbit IgG (H+L) antibody (Zymed Laboratories Inc, San Francisco, CA), radiolabelled with Iodogen as described [15], was added and incubated at 20 °C for 2 h. After extensive washing, the radioactivity in each tube was counted in a well-type gamma counter (Aloka, Tokyo, Japan).

The sensitivity of the IRMA was about 0.5 ng/ml, and the intra- and inter-assay coefficients of variation were 2.8% (n=8) and 4.2% (n=4) at a concentration of 10 ng/ml. The recovery rate of 10 ng/ml of rh-follistatin added to the culture medium was 90–105%. The addition of 16–32 molar excess of rh-activin A to the same sample did not affect the follistatin recovery rate, indicating that this IRMA can measure the total level of activin-bound and -unbound follistatin. There was no significant cross-reaction with rh-activin A, porcine activin B, bovine inhibin, rFSH, rLH and rGH up to 500 ng/ml in the present assay.

Western blotting

The culture media collected from anterior pituitary cells cultured for 9 days were pooled (total
volume 40 ml) and condensed by means of affinity chromatography with 0.5 ml of sulfate-cellulofine gel (Seikagaku Corporation, Tokyo, Japan) that was blocked overnight with 5% FCS-DMEM [16]. After washing with FCS-free DMEM and 0.05 M phosphate buffer, pH 7.4, containing 0.3 M NaCl, the sample was fractionated with 0.5 ml of 1% sodium dodecyl sulfate (SDS). The fractions were loaded onto SDS-polyacrylamide gels (14%) and separated at a constant current of 30 mA for 6 h, then electrotransferred to a 0.45 μm nitrocellulose membrane. The membrane was quenched with 2% Block Ace in 0.02 M Tris-buffered saline (TBS), pH 7.4, at 4 °C for 24 h, washed with TBS containing 0.2% Tween-20 at 20 °C for 30 min and incubated with rabbit anti-follistatin polyclonal antibody (1:600) in 0.025 M TBS containing 0.4% Block Ace, 1% normal goat serum and 0.2% Tween-20 at 20 °C for 2 h. The nitrocellulose membrane was further washed for 60 min, and incubated with the second antibody (goat anti-rabbit IgG coupled to horseradish peroxidase, 1:3000, Zymed Laboratories, USA) at 20 °C for 60 min. After incubation with ECL Western blotting detection reagents (Amersham International plc, UK), the membrane was exposed to X-ray film for 5 min. Non-specific interactions were routinely monitored by incubating parallel samples with normal rabbit serum or with preabsorbed antiserum instead of anti-follistatin rabbit polyclonal antibody.

Statistical analysis

Values are expressed as the means ± SD of six replicates. The results were subjected to analysis of variance, and statistical significance between the groups was assessed by Student's t test when the variation of the data was uniform.

Results

Basal secretion of ir-follistatin from cultured rat anterior pituitary cells

The ir-follistatin release into the culture medium from the anterior pituitary cells was detected by IRMA and the accumulated ir-follistatin levels in the culture media gradually increased (1–18 days) as shown in Fig. 1. The ir-follistatin level reached its maximum at 12–15 days with an approximate value of 3.7–5.6 ng/10^6 cells in two independent experiments.

In cultures with densities from 0.25–4 × 10^6 cells/ well, the increase in basal ir-follistatin secretion was all density-dependent after 6 days in culture with 2% FCS-DMEM (Fig. 2A). The basal ir-follistatin release into the culture medium was also closely correlated with the FCS concentration in the culture medium and increased 4-fold with 2% FCS-DMEM (P< 0.01) and 6-fold with 10% FCS-DMEM (P< 0.001) compared with 0% FCS-DMEM (Fig. 2B).

Effects of LHRH, FSH and activin A on ir-follistatin secretion from the pituitary

As shown in Fig. 3, the addition of LHRH and rh-activin A resulted in a significant increase in ir-follistatin accumulation in the culture medium. LHRH (100 nM) and rh-activin A (0.5 and 5 nM) induced about 2- (P< 0.01) and 1.8-fold (P< 0.05) increases in ir-follistatin accumulation, respectively. In contrast, FSH (0–4 nM) did not significantly affect ir-follistatin release (Fig. 3A). LHRH (100
nM) and rh-activin A (0.5 nM) stimulated ir-follistatin release into the culture medium in a time-dependent manner (Fig. 3B).

Molecular heterogeneity of follistatin in the culture medium

Immunoblotting with anti-follistatin polyclonal antibody showed no detectable cross-reactivity with rFSH, rLH, rh-activin A and porcine activin B at a concentration of 1 µg/ml (data not shown). As shown in Fig. 4, the rh-follistatin standard used in this study showed at least three different sharp bands ranging from 35 to 45 kDa. Several protein bands (over 200 k, 95–100 k and 35–50 kDa) were identified in the condensed medium from cultured pituitary cells, and the condensed medium without cultured cells contained a protein band of over 200 kDa with a similar intensity and protein bands from 35 to 50 kDa with lower intensity. These proteins were not identified in the control study with non-immune serum or preabsorbed antiserum (data not shown).
Discussion

There is accumulating evidence that activin is produced in the pituitary and acts as a local regulator for FSH secretion [6, 8, 17–19]. The follistatin gene is also expressed in various extragonadal tissues including the pituitary and almost invariably coexists with the activin subunit gene [6, 7, 20], suggesting that follistatin plays an important role in modulating activin action within these tissues. In this report, we demonstrated that mature follistatin protein is actually secreted from cultured anterior pituitary cells. The ir-follistatin release from cultured pituitary cells was time- and cell density-dependent and also stimulated by the addition of FCS. Although the precise number of viable cells was not monitored all through the experiments, the viable cells estimated by trypan blue dye exclusion did not increase in number during the culture but rather decreased after 12 days of incubation. Accordingly, ir-follistatin release from cultured pituitary cells can also be affected by some serum factors regardless of cell density.

When dispersed rat anterior pituitary cells were incubated with rh-activin A, the ir-follistatin level in the culture medium increased in a dose-dependent fashion. This result coincides with the finding that activin A stimulates follistatin production from cultured rat anterior pituitary cells [12], supporting the notion of a local circuit regulated by activin and follistatin in the pituitary. LHRH also significantly enhanced ir-follistatin release from cultured rat anterior pituitary cells. Although the mechanism remains unclear, this stimulatory effect of LHRH on ir-follistatin release may be mediated by pituitary activin. We also found that ir-activin A secretion is significantly enhanced by LHRH in cultured rat anterior pituitary cells [21]. Furthermore, DePaolo et al. demonstrated that pituitary follistatin expression is enhanced after castration by the mediation of pituitary activin [22]. Other investigators have also revealed that the pituitary follistatin mRNA level varies during the rat estrous cycle and increases remarkably after castration in relation to an LHRH pulse [23, 24]. These data suggest that follistatin production is closely related to pituitary activin and that LHRH is one of the positive regulators of this system.

The direct effect of FSH on ir-follistatin release was also examined in this study, but we could not find any significant effect. There has been no direct evidence for the presence of FSH receptor in the pituitary, but we recently demonstrated that pituitary activin A secretion is suppressed by the addition of FSH [21], implying the possibility that FSH may also exert a form of direct regulation in the pituitary, but further investigation is necessary to prove this hypothesis.

On the other hand, immunoneutralization with an anti-follistatin antibody produces no significant changes in basal FSH secretion in vitro [12] and the administration of follistatin does not affect the basal and LHRH-induced release of FSH in vivo [25]. We therefore postulate that follistatin does not directly affect the FSH secretion but suppresses it only by neutralizing the stimulatory effect of activin on FSH secretion. It is necessary to further study the effects of gonadal steroids and inhibin on follistatin secretion and the interaction of these local factors.
Native follistatins consist of different forms as a result of truncation and glycosylation [3–5], but the physiological role of each form and its distribution in vivo are unknown. Sugino et al. have demonstrated that FS-315 and FS-288 have a common feature in binding to activin but have a different chemical nature, suggesting that their physiological functions differ [5]. Immunoblotting of the condensed medium from cultured rat anterior pituitary cells with anti-follistatin antibody revealed three distinct follistatin bands ranging from 35 to 50 kDa, among which an immunoreactivity around 50 kDa was the most intense. Recently, at least three major follistatins ranging from 31 to 39 kDa have been purified from bovine pituitaries [10, 26], and two broad bands corresponding to 35–46 and 39–53 kDa were visualized in the medium of cultured rat anterior pituitary cells under non-reducing and reducing conditions, respectively [12]. Our results were generally in agreement with these findings. These data including ours indicate that the major form of follistatin released from the pituitary cells is the glycosylated form of FS-315. As the cell-associated short form of follistatin (FS-288) is supposed to effectively capture activin on the cell surface and modify its binding to the activin receptor, it is also important to investigate the production and regulation of FS-288 as well as FS-315 in the pituitary. As a protein band over 200 kDa in the condensed culture medium was similarly recognized in the control medium, it may represent non-specific binding with large proteins in the serum.

In conclusion, we demonstrated that ir-follistatin is actually secreted from cultured rat anterior pituitary cells and that its secretion is increased at least by activin and LHRH. Further investigation is necessary to clarify the roles and mechanisms of the activin/follistatin system in the pituitary gland.

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References


