Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related  

(radiusinoassay/rats/immunoaffinity)

Shao-Yao Ying, Joseph Czvik, Ann Becker, Nicholas Ling, Naoto Ueno, and Roger Guillemin

Laboratories for Neuroendocrinology, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT The production of inhibin in cultured granulosa cells from immature hypophysectomized, estrogen-treated rats and Sertoli cells from normal animals was determined by a specific radioimmunoassay using an antiserum against a synthetic replicade of [Tyr30]inhibin α-chain-(1-30). The amount of immunoreactive inhibin detected in the spent media of these cells is in proportion to the density of cells plated and the concentration of exogenously added follicle-stimulating hormone (FSH). In the presence of the estrogen precursor androstenedione (10^-7 M), FSH, but not luteinizing hormone, produced a dose-dependent increase in inhibin during 2-day culture of granulosa cells. In the absence of the estrogen precursor, similar but somewhat diminished inhibin production in responding to FSH was observed. Exogenously added estrogen potentiated the FSH-mediated release of inhibin in the absence of androstenedione. Neither androstenedione nor estradiol added to the cultured Sertoli cells had effect on inhibin production. A preparation of pure inhibin isolated on the basis of an in vitro bioassay and characterized chemically specifically suppressed serum FSH but not luteinizing hormone, when it was injected (24 μg per injection, two injections) into acutely ovariectomized rats. Thus, inhibit secreted by the granulosa and Sertoli cells specifically suppresses the secretion of pituitary FSH, and in turn FSH is primarily responsible for the inhibin production in these gonadal cells, as in a classical negative-feedback relationship.

Two forms of porcine inhibin (IN) were recently isolated from follicular fluids, partial amino-terminal sequences were determined by chemical (1-3), and the total amino acid sequences of the mature proteins and their precursors were deduced from complementary DNA sequences (4, 5). INs are proteins of Mr = 32,000 consisting of two polypeptide chains linked by disulfide bonds. The two forms (A and B) share one common α chain of Mr, 18,000 and are distinguished by the similar but distinct β chain of Mr, 14,000. IN-A and IN-B are statistically equipotent as specific inhibitors for the basal secretion of follicle-stimulating hormone (FSH; follitropin) in vitro (2). Similar results were subsequently reported for the characterization of INs of human (6), bovine (7), and murine (8) origins. We report here (i) evidence of the release of immunoreactive IN (ir-IN) by rat granulosa cells and Sertoli cells in vitro as determined by radioimmunnoassay (RIA) and show it to be dependent on cell density and FSH dose; (ii) that an antiserum raised to the synthetic peptide [Tyr30]IN-α-(1-30) neutralizes the IN activity in vitro as well as elevates the plasma FSH levels in vivo, a result best explained by neutralization of endogenous IN by the antiserum; and (iii) that samples of IN-A as obtained after the in vitro assay, in the highly purified form used to generate amino acid sequence data, are active in vivo to suppress FSH secretion in the rat. Thus, a reciprocal relationship (negative feedback) between the secretion of FSH and that of IN is established, based on in vitro and in vivo results. To our knowledge, there are no previous data demonstrating the inhibition of FSH release in vivo by IN-A in pure form.

MATERIALS AND METHODS

Antiserum Development. [Tyr30]IN-α-(1-30) was synthesized by solid-phase methods (9). The synthetic replicate was conjugated to bovine serum albumin by bisdiazotized benzidine as described previously (10). Three-month-old male New Zealand White rabbits were injected intradermally at multiple sites with 1 mg of the peptide conjugate emulsified in Freund’s complete adjuvant (Difco) containing extra desiccated Mycobacterium tuberculosis at 1 mg/ml. In addition, a subcutaneous injection of 2 ml of denatured Bordetella pertussis (10^7 organisms per ml) was given at initial injections. Animals were boosted regularly at 2- to 4-wk intervals, using 200 ng of the conjugate emulsified in Freund’s incomplete adjuvant.

RIA for IN. Synthetic [Tyr30]IN-α-(1-30) was radioiodinated by the method of Greenwood et al. (11) with slight modifications. The reaction mixture was applied to a Sephadex G-10 column (1.0 × 20 cm) equilibrated with 30% (wt/vol) acetic acid. The 125I-labeled peptide, 125I-[Tyr30]IN-α-(1-30), was diluted and used for RIA.

After the third and subsequent boosts, the rabbits were bled and sera were examined for their capacity to bind 125I-[Tyr30]IN-α-(1-30). The antisera found to bind 30-50% of the radioiodinated peptide at final dilutions of 1:25,000 to 1:100,000 were used in displacement studies, with native purified porcine IN-A as the unlabeled antigen. All incubations were done in RIA buffer (30 mM Hepes/150 mM NaCl/0.1% NaN3/0.1% bovine serum albumin, pH 7.6) in disposable borosilicate glass tubes. Serial dilutions of the HPLC-purified IN-A in 100-μl aliquots, the antiserum of predetermined dilution in 200 μl, and 125I-[Tyr30]IN-α-(1-30) in 100 μl (10,000 cpm) were added. The tubes were incubated at room temperature for 24 hr and the bound and free antigens were separated by using Staphylococcus aureus protein A or goat anti-rabbit gamma globulin. The mixtures were centrifuged for 30 min in a Beckman model J-6B centrifuge, the supernatants were aspirated, and the radioactivities of the pellets were measured for 1 min on a γ counter.

Culture of Rat Granulosa and Sertoli Cells. Granulosa cells were prepared as described (12). Briefly, ovarian follicles of diethylstilbestrol-treated, hypophysectomized immature rats were punctured and cells were squeezed out gently with two hypodermic needles. The cells were washed, plated in

Abbreviations: IN, inhibin; FSH, follicle-stimulating hormone (follitropin); ir-IN, immunoreactive inhibin; LH, luteinizing hormone (lutropin); o-, ovine; PFF, porcine follicular fluid.

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McCoy’s medium, and treated as described in the legends to the figures. The amounts of ir-IN secreted into the medium in 48 hr were measured by RIA. Fractions enriched in Sertoli cells were prepared from rat testes according to the method described by Mather et al. (13) with slight modification. Briefly, testes obtained from 22-day-old male Sprague–Dawley rats were decapsulated, the Leydig cells were separated by collagenase dispersion at room temperature for 7 min, and then the testicular tissues were further dispersed at 37°C for 25 min. The digested tissue was then filtered through 30-μm mesh nylon gauze, the retentate being the fraction enriched in Sertoli cells.

**Immunaoaffinity Chromatography of Conditioned Medium.** Medium (220 ml) from 48-hr cultured rat granulosa cells was pooled and passed through an immunooaffinity column (8 ml), prepared by coupling purified IgG raised against [Tyr30]IN-A (1-30) to Affi-Gel 10 (Bio-Rad) according to the manufacturer’s recommendations. After the unbound material had been washed off with Dulbecco’s phosphate-buffered saline (PBS), the absorbed ir-IN was eluted with 0.1 M HCl. Aliquots (50 μl) of the eluate were brought to pH 8.00 with 10 μl 1 M Tris buffer, pH 8.00, and the ir-IN was measured by RIA.

**Neutralization of IN with the Antiserum.** For *in vitro* studies, antiserum was added concurrently with various concentrations of purified IN in the bioassay for IN-A, using cultured rat anterior pituitary cells as described previously (14). Cells were enzymatically dispersed and plated for 24 hr, then antiserum or normal rabbit serum with or without various doses of IN was added for 2 more days. The media were removed and FSH was measured. For *in vivo* studies the antiserum was injected into intact metaestrous rats and serum was collected 8 hr after injection and measured for FSH. Normal rabbit serum was used as the control in both cases. Two such *in vivo* experiments were conducted. FSH was measured by employing an RIA kit provided by the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (one using as reference standard FSH-RP-1, the other the latest reference standard, FSH-RP-2, as our supply of RP-1 became exhausted).

**Inhibition of the Secretion of FSH by Purified IN in Vivo.** Mature female Sprague–Dawley rats were obtained from Charles River Breeding Laboratories and housed at a temperature of 25°C and a light regimen of 14 hr of light and 10 hr of darkness (midnight = middle of dark period). Food and

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**Fig. 1.** (A) Displacement of [125I-Tyr][30]IN-A (1-30) binding to antisera raised against synthetic [Tyr][30]IN-A (1-30) by synthetic [Tyr][30]IN-A (1-30) (c), native IN-A (c), and native IN-B (m). When [125I-Tyr][30]IN-A (1-30) was used as the radioligand amounts of [Tyr][30]IN-A (1-30) as low as 1 pg/ml were detectable, and half-maximal displacement was found at an IN-A (1-30) concentration of 150 pg/ml (100 fmol/ml). However, 7.2 ng/ml (225 fmol/ml) purified native IN was needed to show 90% competition with [125I-IN-A (1-30) for the antisera, with a minimal detectable dose as low as 130 pg/ml (4 fmol/ml) and a half-maximal displacement at 1.85 ng/ml (58 fmol/ml). No displacement was seen with FSH, LH, or thyrotropin-stimulating hormone (TSH) at 500 ng/ml; prolactin (PRL) or growth hormone (GH) at 20 ng/ml; highly purified relaxin, activin (purified in this laboratory), or transforming growth factor β (TGFβ) at 100 ng/ml; or the following synthetic fragments of IN at 100 ng/ml: α chains (1–7), (39–58), (97–116), (117–130), and (97–130) or β chains (66–79), (58–77), (38–59), (47–78), (97–106), (82–93), and (45–66). (B) ir-IN in the spent media of cultured rat granulosa cells. Granulosa cells (5 × 10^4 cells per ml) were incubated with FSH at 0.5–2 ng/ml for 48 hr in the presence of 10^{-7} M androstenedione. Each value represents the mean ± SD of duplicates. (C) Measurement of ir-IN in the spent media of cultured rat granulosa cells and rat Sertoli cells. Each value represents the mean ± SD of triplicates. (D) The behavior of ir-IN in conditioned medium from rat cultured granulosa cells that eluted from an immunooaffinity column (Right) was parallel to that of native IN purified from PFF (Left) in the RIA dose-response measurement. Each point represents the mean of duplicates.
water were supplied ad lib. Vaginal cytology was examined daily; rats having 4-day cycles and exhibiting at least three consecutive cycles were used in the experiments. Rats were ovariectomized between 0800 and 0900 on metaestrus and received i.p. injections of purified IN-A of various doses 1 min and 4 hr after the operation. IN-A was purified from porcine follicular fluid (PFF) as described (2). As internal control, a group of rats received 0.25 ml of crude PFF in the same regimen. Eight hours after ovariectomy, trunk blood was collected by decapsulation for measurement of FSH and LH (luteinizing hormone) by RIA as antisera obtained from the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. NIH-LH-RP-1 and NIH-FSH-RP-2 are the standards in RIA, respectively.

**Statistical Analysis.** Statistical significance of experimental results was determined by the multiple comparison test of Duncan and Dunnett. The dose–response curves were calculated according to the best-fit program (Allfit) described by De Lean et al. (15). Potencies were determined by the Bioprog method described by Rodbard (16). All calculations were made through the Biocomputing Laboratory of The Salk Institute.

**RESULTS**

The amount of ir-IN measured in the medium of cultured rat granulosa cells, in the presence of $10^{-7}$ M androstenedione, can be linearly increased by treatment of the cells with increasing concentrations of ovine FSH (oFSH) (Fig. 1B), as determined by the highly sensitive, specific RIA for IN using antisera raised against a synthetic peptide of [Tyr]$^{30}$IN-$\alpha$-(1-30) (Fig. 1A) (described above). A similar dose-dependent increase in the release of ir-IN was observed in cultured rat Sertoli cells treated with oFSH (Fig. 1C). This FSH-mediated release of ir-IN in the spent medium of cultured granulosa and Sertoli cells is also cell density dependent (data not shown). In contrast to the effects of FSH, similar doses of highly purified ovine LH (oLH) devoid of FSH were not effective (data not shown).

To investigate further the nature of the ir-IN detected in the tissue culture of granulosa cells, it was compared with a partially purified porcine IN on an immunofluorescence chromatography substrate. Like the native IN, the released form of ir-IN is retained on the immunoaffinity column and eluted with 0.1 M HCl. Dilution curves with the fractions eluted by 0.1 M HCl show a parallel and dose-dependent displacement that is otherwise indistinguishable from that obtained with purified native IN (Fig. 1D) from PFF.

In the absence of the estrogen precursor $\Delta^4$-androstenedione, cultured granulosa cells showed typical, although somewhat diminished, IN-producing responses to FSH over a dose range of $0.5-8 \mu g/ml$. Various doses of $17\beta$-estradiol ranging from $10^{-12}$ to $10^{-6}$ M added exogenously to these cells showed no IN-producing activity (data not shown). However, 2 ng/ml of $17\beta$-estradiol ($7 \times 10^{-9}$ M), a dose usually measured in cells showing FSH-induced estrogen accumulation from the precursor, added to cells with no estrogen precursor, did further enhance IN secretion induced by FSH. Indeed, the amount of IN produced was not significantly different from that in cells in the presence of androstenedione (Fig. 2).

The antisera (1 $\mu l/ml$) added to cultured rat pituitary cells completely neutralized the bioactivity of IN with slight residual activity at a very high dose of IN (8 ng/ml) (Fig. 3). The minimal effective dose of the antisera to neutralize an effective dose of IN (2 ng/ml) in vitro is <0.2 $\mu l$ (data not shown). In two experiments, administration of 1 ml of antisera to metaestrous rats resulted in an 8-fold increase in serum FSH compared to control rats treated with normal rabbit serum (P < 0.01); 819 ± 73 (n = 10) vs. 100 ± 2.4 (n = 11) (results of the two experiments are expressed in percentage variations from the control group rather than in absolute values, because the reference standard for FSH in the RIA was NIH-FSH-RP-1 for the first experiment and NIH-FSH-RP-2 for the second experiment).

Two injections of purified porcine IN-A each at a dose of 24 $\mu g$ led to a decrease of serum FSH levels from 10.49 ± 0.24 to 2.70 ± 0.4 ng/ml (P < 0.01) (Fig. 4). In the same experiment this suppression of serum FSH was comparable to that of two injections of 0.25 ml of crude PFF (down to 3.70 ± 0.30 ng/ml). Doses of 12 $\mu g$ showed a slight decrease that was not statistically significant. Serum LH levels were not affected by either purified IN or PFF.

**DISCUSSION**

We report here that antisera raised against a synthetic replicate of the N-terminal sequence [Tyr]$^{30}$IN-$\alpha$-(1-30) of the common $\alpha$ chain of porcine IN neutralize the biological activity of IN in vitro and elevate the serum FSH level in vivo when injected in metaestrus rats. We have also established, using a RIA for rat IN developed with the same antisera, that IR-IN can be detected in the culture medium of rat granulosa and Sertoli cells. This release of IN is increased by oFSH in a dose- and cell density-dependent manner; highly purified oLH is inactive in this respect. The effect of FSH in

![Fig. 2. Effect of steroids on the induction of ir-IN by FSH in cultured granulosa cells.](image)

**Fig. 2.** Effect of steroids on the induction of ir-IN by FSH in cultured granulosa cells. •, Δ4-androstenedione at $10^{-7}$ M; ○, 17β-estradiol at $7 \times 10^{-9}$ M; ■, no steroids.

![Fig. 3. In vitro neutralization of IN by an antisera raised to [Tyr]$^{30}$IN-$\alpha$-(1-30).](image)

**Fig. 3.** In vitro neutralization of IN by an antisera raised to [Tyr]$^{30}$IN-$\alpha$-(1-30). ○, Control, no antibodies; •, normal rabbit serum; ■, IN antisem. Data points represent the mean of results obtained in three wells; vertical bars represent SEM.
stimulating the secretion of ir-IN is potentiated by endogenous or exogenous estrogens (see below). Bioactive IN in the conditioned media of rat granulosa and Sertoli cells has been previously reported (17–20), and Lee et al. (21) established that pregnant mares’ serum gonadotropin, with primarily FSH activity, stimulated the release of bioassayable IN. Bicsak et al. (22) have recently reported that several hormones and agents that are known to stimulate aromatase activity in granulosa cells also stimulate IN production as determined by RIA using antisera to a synthetic replicant of the N-terminus of the α chain of IN, [Gly<sup>26</sup> Tyr<sup>27</sup>]IN-α (1–27). In contradiction to some of our results, they reported that two preparations of FSH and LH (NIH-FSH-S16 and NIH-LH-S23) stimulated IN production in FSH-primed granulosa cells (cells were cultured in the presence of FSH for 2 days followed by treatment with increasing concentrations of FSH or LH for an additional 3 days). This LH preparation contains >0.5% FSH activity by weight according to the data sheet provided by the National Institutes of Health. In our hands, highly purified LH, which has no intrinsic FSH activity, does not stimulate IN production in granulosa cells cultured for 2 days. In such a 2-day incubation regimen, highly purified oLH in the presence of FSH neither further produces IN over and above that stimulated by FSH nor negates IN secretion induced by FSH, nor does highly purified oLH have any effect on IN production in cultured rat Sertoli cells. The lack of IN-producing activity in LH was recently observed by Bicsak et al. (23) in cultured rat Sertoli cells.

The present study demonstrates further the ability of FSH, even in the absence of the estrogen precursor Δ<sup>5</sup>-androstenedione, to stimulate IN secretion in granulosa cells. Moreover, estradiol alone was inactive in this respect, thus indicating that estrogen can be ruled out as the mediator of FSH-induced IN production. It also suggests that FSH directly acts on granulosa cells to stimulate IN secretion. Further data indicate, however, that estrogens, either as biosynthesized from the Δ<sup>5</sup>-androstenedione or exogenously added, potentiate the effects of FSH on stimulating the production of IN.

As presented here, antiserum raised against [Tyr<sup>30</sup>]IN-α-(1–30) neutralizes the biological activity of IN in vitro as well as that of endogenous IN in vivo. Rivier et al. (24) have reported that antiserum to [Glu<sup>26</sup> Tyr<sup>27</sup>]IN-α-(1–27) injected in cycling rats on diestrus of the estrous cycle neutralized endogenous IN as demonstrated by elevated plasma FSH levels. Our in vivo immunoneutralization results are in agreement with their observations.

The data obtained in vivo that purified IN specifically inhibits secretion of serum FSH validate the in vitro method used in the purification and isolation of IN. The effective dose of porcine IN-A used here in vivo (2 × 25 μg or 2 × 0.75 nmol) may appear to be high. This is not without precedent. For example, 2–5 μg (1–4 nmol) of somatostatin is considered as the minimal effective dose to inhibit production of plasma growth hormone (GH) in pentobarbital-treated rats (25), whereas a dose of 50 ng (∼0.01 nmol) of GH-releasing factor is adequate to stimulate secretion of GH in anesthetized rats (26). Thus, it is not unreasonable that the injection of 2 × 24 μg (5.76 nmol) of purified IN (2 × 75 nmol) into acutely ovarietomized rats is required to inhibit FSH secretion as judged by serum FSH levels. Also, this dose of IN-A may appear to be high compared with the volume of crude PFF producing the same biological effects. Several forms of INs have been observed in follicular fluid by us and other groups during the process of estrus (2, 7); all are biologically active, and it is possible that the higher molecular weight form of INs (∼56,000) has a (yet-to-be-determined) longer biological half-life than the M<sub>r</sub> 32,000 IN-A used here. Because only very limited quantities of highly purified native porcine IN were available, doses higher than 24 μg could not be tested in these studies. However, the in vivo results presented here provide evidence that the INs isolated and characterized chemically and through methods of molecular biology are the molecules that account for the IN activity in PFF.

These studies show that IN acts directly upon the anterior pituitary specifically to decrease the output of FSH. In turn, the circulating levels of FSH determine the production of IN in the gonad as in a classic negative-feedback mechanism.

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