

Recombinant human activin-A stimulates basal FSH and GnRH-stimulated FSH and LH release in the adult male macaque, *Macaca fascicularis*.

Robert I. McLachlan, Kristine D. Dahl, William J. Bremner, Ralph Schwall, Charles H. Schmelzer, Anthony J. Mason, and Robert A. Steiner

Departments of Medicine (RIM, KDD, WJB), Obstetrics and Gynecology (RAS), Physiology and Biophysics (RAS), Zoology (RAS), The Population Center for Research in Reproduction, and the Regional Primate Center at the University of Washington, Seattle, WA 98195; the Veterans Administration Medical Center, Seattle, WA 98108; and the Departments of Recovery Process Research and Development, and Developmental Biology, Genentech Inc. (RS, CHS, AJM), South San Francisco, CA 94080.

Abstract: Activin-A is a homodimer of the β_h inhibin subunit that stimulates FSH secretion by pituitary cells *in vitro*; however, the physiological relevance of this effect is unknown. We have examined whether recombinant human activin-A (activin-A; 80 $\mu\text{g/kg/day}$ iv infusion for 50.5 h) has *in vivo* bioactivity in the adult male macaque ($n=5$). Serum FSH and LH bioactivity and serum testosterone (T) levels were measured on 2 control days and after 24 and 48 h of activin-A administration. Basal FSH levels increased significantly ($p<0.05$) by 17% at 24 h and 82% at 48 h during activin-A administration. No changes in basal LH or T levels were seen. The FSH and LH responses to GnRH (5 $\mu\text{g/kg}$, iv bolus) increased significantly ($p<0.05$) by 117% and 55% after 48 h of activin-A, respectively. A small (16%), but statistically significant ($p<0.05$), increase in the T response to the GnRH challenge was also noted. These data are preliminary evidence in support of a physiological role for activin-A in the control of gonadotropin secretion in the male primate.

INTRODUCTION

The activins are dimers of the β subunits of inhibin (1,2) which were isolated in two forms, activin A ($\beta_h\beta_h$ dimer) or activin AB ($\beta_h\beta_a$ dimer), based on their ability to stimulate the release and synthesis of FSH by pituitary cells in cultures. These actions are antagonized by inhibin under both basal and GnRH-stimulated conditions (1). Subsequently, a wide range of other activities have been ascribed to the activins, including stimulation of hematopoiesis (3,4), immunoregulation (5), and paracrine regulation of ovarian (6) and testicular function (7).

Recently, recombinant human activin-A has been expressed and purified and shown to have *in vitro* activities similar to that of the isolated native molecule (8,9). We used this recombinant material to examine whether activin modulates gonadotropin release in the adult male macaque. We report here the effects of a 2 day infusion of activin-A on basal and GnRH-stimulated gonadotropin and testosterone secretion, and offer preliminary evidence to suggest a physiological role for circulating activin-A in the male of a primate species.

MATERIALS AND METHODS

Animals and surgeries

Five adult male crab-eating macaques, *Macaca fascicularis*, (weighing 4-7 kg) were housed under controlled conditions of temperature ($21\pm 2^\circ\text{C}$) and light (on at 0600h; off at 1800h) in individual cages in the Regional Primate Research Center at the University of Washington. In addition to monkey chow, the animals received fresh fruit, chewable vitamins and iron injections.

Three days prior to the study, an indwelling femoral venous catheter was inserted under fluothane anesthesia. The distal portion of the catheter was run subcutaneously around the flank, exteriorized in the midscapular region and run through a protective housing to a sampling port on the top of the cage. Patency of the catheter was maintained by constant slow infusion of heparinized normal saline. This arrangement allowed blood sampling from unanesthetized free-moving animals.

Drugs

Recombinant human activin-A (activin-A) was produced and purified as described (9) and stored at 4°C in 0.1 M acetic acid. Approximately 300 μl of activin-A was diluted in 55 ml of normal saline containing 1.6% normal male macaque serum (included as a carrier protein) and delivered at 4.1 ml/hour by

a Harvard infusion pump. Infusion solutions were changed every 12 h. No adverse effects were seen during activin-A administration. Gonadotropin-releasing hormone (Factrel, Ayerst Lab. Inc., New York) was diluted in sterile normal saline.

Hormone Assays

Bioactive LH and FSH levels in serum samples were measured by *in vitro* Leydig cell (10) and granulosa cell (11) bioassays, respectively. A human pituitary gonadotropin preparation (LER-907; FSH biopotency 20 IU/ μg ; LH biopotency, 60 IU/mg by the 2nd IRP of human menopausal gonadotropin standard) was used as the standard reference preparation for both bioassays. Serum bioactive FSH from each individual animal was analyzed in triplicate at three dose levels (5, 10 and 20 μl). At the end of the 3-day culture period, medium was analyzed for estrogen content by RIA (Diagnostic Products Corp., Los Angeles, CA). The sensitivity of the FSH bioassay was 1.5 ng/culture well with intra- and interassay CVs of 10 and 14%, respectively. The sensitivity of the LH bioassay was 0.15 ng/tube with intra- and interassay CVs of 10 and 22%, respectively. Testosterone levels were measured after a 3-hour incubation (Leeco Diagnostics Inc, Southfield, MI).

To determine whether the presence of activin-A in the serum samples would influence the performance of the FSH and LH bioassays, we performed dose-response curves of the gonadotropin standard in the presence of a wide range of activin-A concentrations, encompassing the maximum theoretical concentration that could have been achieved in serum (i.e., assuming no metabolism of the infused activin-A). This was necessary since we are currently unable to measure activin-A in serum or to estimate its serum half-life. Rat granulosa and mouse Leydig cells were cultured with increasing concentrations of activin-A (3 to 1000 ng/culture) with or without increasing concentrations of the human pituitary gonadotropin preparation (LER-907; Figure 1). In the FSH bioassay, LER-907 stimulated estrogen production in a dose-dependent manner, with a minimal detectable limit of 1.5 ng/well. Addition of increasing concentrations of activin-A resulted in FSH dose-response curves parallel to the standard curve (Figure 1A). However, activin-A concentrations above 30 ng/ml significantly stimulated estrogen production, regardless of the FSH concentration, with the effect being maximal at ≥ 300 ng/ml. Stimulation of granulosa cell estrogen production by native activin-A has been reported previously (6). Activin-A (1000 ng/ml) was added to each tissue culture well to compensate for this effect of activin-A on estrogen production. Activin has also been shown to inhibit hCG-stimulated T production by Leydig cells; however, this effect has a delayed onset of 12-24 h (7). The LH bioassay used in the study involved only a 3 h incubation. When increasing concentrations of activin-A were added with LER-907 to the LH bioassay, there were no significant

Address all correspondence to Robert A. Steiner, Department OB/GYN RH-20, University of Washington, Seattle, WA 98195, (206) 543-8712.

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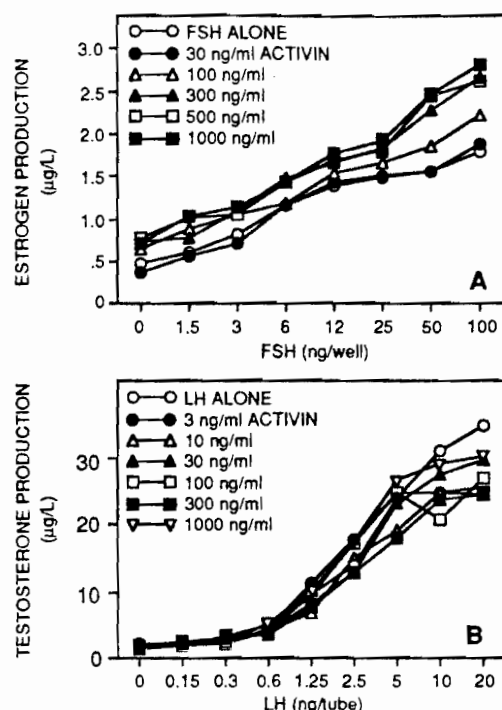


Figure 1. Effects of activin-A on the FSH and LH Bioassays. Rat granulosa cell cultures (top panel) and mouse Leydig cell cultures (bottom panel) were incubated with increasing concentrations of a human pituitary preparation (LER-907) together with increasing concentrations of activin-A. E production after a 3-day culture and T production after a 3-hour incubation were analyzed by RIA for the FSH and LH bioassays, respectively. Data are presented as mean \pm SE of triplicate cultures.

changes in T production (Figure 1B), and therefore activin-A was not included in this assay.

The reagents for the serum testosterone RIA were provided by the WHO Matched Reagent Programme and involved ether extraction of serum and separation of bound from free T by dextran-coated charcoal (12). The T assay sensitivity was 0.35 nmol/L and the intra- and interassay CVs were 5.1% and 9.5%, respectively.

Experimental Design

Control Period (days 1-3). Blood samples (1.3 ml) were obtained every 30 min on day 1 between 0700-1000 h, following which a bolus of GnRH (5 μ g/kg, iv) was given and further samples collected 15, 30, 45, 60, 90 and 150 min later. On day 3, another set of baseline samples were obtained every 30 min between 0700-1000 h.

Activin-A infusion (days 3-5). Immediately following the 1000 h sample on day 3, a bolus of activin-A (10 μ g/kg, iv) was given and a 50.5 h infusion of activin-A begun (80 μ g/kg/day). Blood samples were obtained every 30 min between 0700-1000 h on day 4 and day 5 (i.e., after 24 and 48 h of activin-A). Immediately after the 1000 h sample on day 5, a bolus of GnRH (5 μ g/kg, iv) was given and samples collected 15, 30, 45, 60, 90 and 150 min later, with the last sample coinciding with the end of the activin-A infusion. The activin-A infusion was interrupted momentarily to accomplish the blood sampling.

Blood samples were allowed to clot overnight at 4°C, and the serum collected and stored at -20°C prior to assay. Samples from an individual animal were measured in the same assay for serum testosterone, FSH and LH.

Statistical Analysis

Baseline serum levels of FSH, LH and T were assessed by pooling the 14 individual values obtained between 0700-1000 h on days 1 and 3. These values were compared by repeated

measures analysis with the means of the 7 values obtained between 0700-1000 h on days 4 and 5 (after 24 and 48 h of activin-A, respectively). Changes in GnRH-stimulated gonadotropin and T release were assessed by integration of the area under the time-response curve, calculated with Simpson's rule, on days 1 and 5 and comparisons were made by the Wilcoxon's matched pairs test. $P < 0.05$ was considered significant.

RESULTS

Mean baseline hormone values during the control period and following 24 and 48 h of activin-A infusion are shown in Figure 2. Serum FSH bioactivity was significantly ($p < 0.05$) increased by 17 and 82% after 24 and 48 h of activin-A, respectively. No significant changes were observed in either serum LH bioactivity or serum T levels. The effects of activin-A infusion on mean serum FSH, LH and T levels for the 5

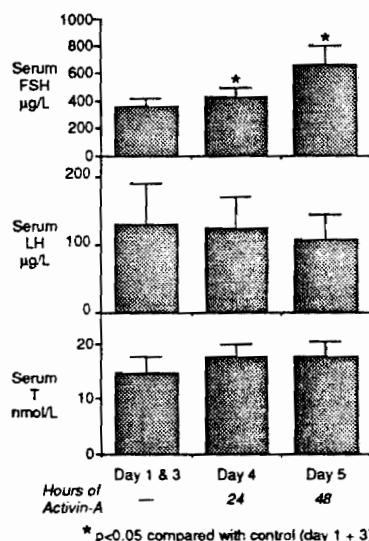


Figure 2. Time course of effects of activin-A in adult male monkeys

animals at each time point are shown in Figure 3.

GnRH-stimulated FSH release was significantly ($p < 0.05$) increased by 117% after 48h of activin-A infusion (Table 1). The mean serum LH and T responses to activin-A also showed significant ($p < 0.05$) increases of 55 and 16%, respectively.

Table 1. Area Under GnRH-Stimulated Response Curves

	Serum FSH μ g 10^3 min/L	Serum LH μ g 10^3 min/L	Serum T nmol 10^3 min/L
Day 1	114 \pm 25	166 \pm 31	16.5 \pm 0.8
Day 5	248 \pm 64*	258 \pm 53*	19.2 \pm 1.7*

* $p < 0.05$

DISCUSSION

We have demonstrated that the infusion of recombinant human activin-A into adult male macaques increased baseline serum FSH levels and augmented the FSH and LH responses to GnRH. No effect was seen on baseline levels of serum LH bioactivity. The stimulation of FSH secretion by activin-A is consistent with the reported action of isolated native activin on pituitary cells *in vitro* (1,2). The stimulation of basal FSH secretion was apparent at 24 h but was more marked at 48 h (approximately doubling); again, this time course and extent of stimulation is consistent with *in vitro* data (1).

A significant increase in GnRH-stimulated LH release was also observed although it was less than half that for FSH. To

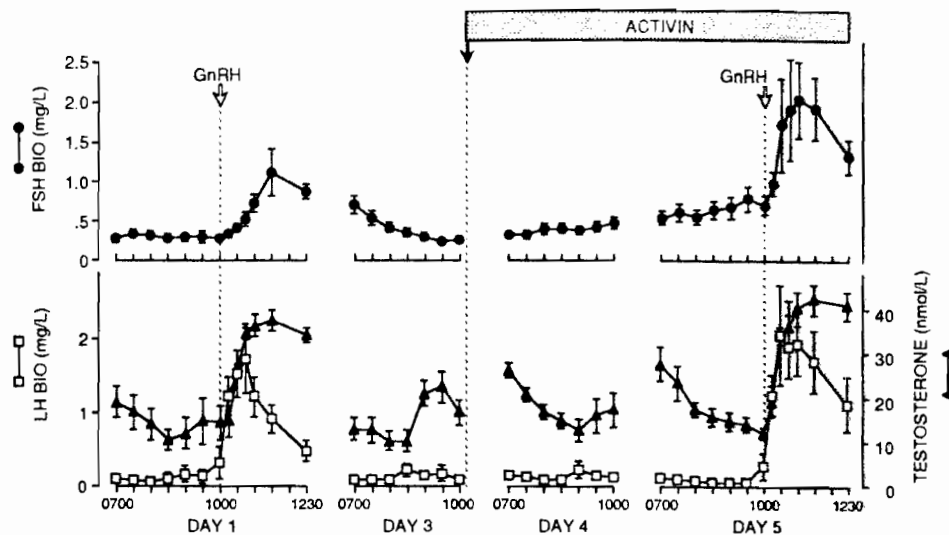


Figure 3. Serum levels of FSH (●) and LH (□) bioactivities and serum T levels (▲) in 5 adult male macaques during the control period (day 1, 3) and activin-A infusion (1000 h day 3 to 1230 h day 5). Activin-A was administered as an initial bolus (10 µg/kg, iv) and then by continuous infusion (80 µg/kg/day). GnRH (5 µg/kg iv stat) was given at 1000 h on day 1 and 5. Data are presented as mean ± SE.

date, there are no reports documenting a significant stimulation of GnRH-induced LH release by activin-A *in vitro* (1). The stimulation we observed may be due to differences in activin action *in vivo*, perhaps due to the dose and/or route of its administration or to other interactions such as antagonism of endogenous inhibin action at the gonadotrope. The effects of activin-A we observed *in vivo* are therefore a mirror image of those described for inhibin *in vitro*, i.e., a predominant, if not exclusive, stimulation of FSH secretion with an effect on LH secretion being most evident with regard to its release by GnRH. A small, but significant, increase in testosterone secretion following GnRH challenge was seen at the end of the infusion. This was probably due to a concomitant increase in GnRH-stimulated LH release.

The recombinant human activin used in this study has been shown to have a biological potency similar to that of the isolated native activin-A in the stimulation of pituitary cell FSH release (9) and stimulation of hemoglobin synthesis in cells (8,9). Recently, an increase in serum FSH levels in female rats has been reported following injection of activin-A (13). The data presented here represent the first direct evidence in support of activin-A having a physiological role as an endocrine signal regulating gonadotropin secretion in the male of any species.

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REFERENCES

- Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J 1986 Purification and characteristics of an FSH-releasing protein from porcine ovarian follicular fluid. *Nature* 321:776.
- Ling N, Ying S-Y, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R 1986 Pituitary FSH is released by a heterodimer of the β -subunit from the two forms of inhibin. *Nature* 321:779.
- Eto Y, Tsuji T, Takezawa M, Takano S, Yokogawa Y, Shibai H 1987 Purification and characterization of

erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochem Biophys Res Commun* 142:1095.

- Yu J, Shao L, Lemas V, Yu AL, Vaughan J, Rivier J, Vale W 1987 Importance of FSH-releasing protein and inhibin in erythroid differentiation. *Nature* 330:765.
- Hedger MP, Drummond AE, Robertson DM, Risbridger GP, de Krester DM 1989 Inhibin and activin regulate (3H) thymidine uptake by rat thymocytes and 3T3 cells *in vitro*. *Mol Cell Endocrinol* 61:133.
- Hutchinson LA, Findlay JK, de Vos FL, Robertson DM 1987 Effects of bovine inhibin, transforming growth factor- β and bovine activin-A on granulosa cell differentiation. *Biochem Biophys Res Commun* 146:1405.
- Hsueh AJW, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW, Vale W 1987 Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc Natl Acad Sci USA* 84:5082.
- Broxmeyer HE, Lu L, Cooper S, Schwall RH, Mason AJ, Nikolics K 1988 Selective and indirect modulation of human multipotential and erythroid hematopoietic progenitor cell proliferation by recombinant human activin and inhibin. *Proc Natl Acad Sci USA* 85:9052.
- Schwall RH, Nikolics K, Szonyi E, Gorman C, Mason AJ 1988 Recombinant expression and characterization of human activin A. *Mol Endocrinol* 2:1237.
- Steiner RA, Peterson AP, Yu JYL, Conner H, Gilbert M, terPenning B, Bremner WJ 1980 Ultradian luteinizing hormone and testosterone rhythms in the adult male monkey, *Macaca fascicularis*. *Endocrinology* 107:1489.
- Dahl KD, Jia X-C, Hsueh AJW, 1989. Granulosa cell aromatase bioassay for follicle-stimulating hormone. *Meth Enzym* 168:414.
- Matsumoto AM, Paulsen CA, Hopper BR, Rebar RW, Bremner WJ 1983 Human chorionic gonadotropin and testicular function: stimulation of testosterone, testosterone precursors, and sperm production despite high estradiol levels. *J Clin Endocrinol Metab* 56:720.
- Schwall R, Schmelzer CH, Matsuyama E, Mason AJ 1989 Multiple actions of recombinant activin-A *in vivo*. *Endocrinology* 125:1420.