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FOLLISTATIN DECREASES ACTIVIN-STIMULATED FSH SECRETION WITH NO EFFECT ON GnRH-STIMULATED FSH SECRETION IN PREPUBERTAL MALE MONKEYS

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ABSTRACT Follistatin is an activin-binding glycoprotein that decreases FSH secretion *in vitro and in vivo* in rats. The mechanism by which follistatin acts is unclear, but it has been suggested that it may bind endogenous activin and neutralize its effects. In this study, we wished to test the ability of follistatin to suppress FSH secretion *in vivo* in primates whose FSH secretion has been stimulated by activin or by GnRH. Six prepubertal male monkeys were injected intravenously with human recombinant follistatin at the dose of 90 μg/kg or 180 μg/kg plus activin (90 μg/kg) or GnRH (10 μg/kg). Frequent blood samples were drawn for 12 hours following each injection. Bio FSH and LH levels were measured in those samples. GnRH and activin each stimulated FSH bioactivity. Both doses of follistatin significantly inhibited the activin-induced increase in FSH ($p<0.05$). The GnRH-induced increase in FSH was not affected by follistatin. LH levels were not affected by follistatin in any of the studies. These data suggest that follistatin can suppress the activin-induced increase in FSH in primates and is consistent with the hypothesis that follistatin can block the physiological effects of endogenous activin in primates. This effect is likely to be due to the binding of follistatin to activin either in the peripheral circulation or at the pituitary level.

INTRODUCTION Three non-steroidal factors, inhibin, activin and follistatin, have been isolated and characterized for their ability to regulate FSH synthesis and secretion (1-10). Inhibin and activin are structurally related peptides that respectively inhibit and stimulate FSH secretion (1-7, 11-15). Follistatin is a glycoprotein hormone, structurally not related to inhibin but also able to suppress FSH (8-10). Recently, human recombinant follistatin has been demonstrated to be a potent suppressor of FSH *in vivo* in the rat, with four times the potency of inhibin in blocking the FSH secretion and with a longer duration of action (16). Follistatin has been reported to bind activin and (with lesser affinity) inhibin, probably through the β subunit (10, 21). Although the physiological meaning of the binding of follistatin to inhibin is unclear, the binding of follistatin to activin results in an inactive complex. In cultures of pituitary, erythroblastic, osteoblastic, neural and granulosa cells the addition of follistatin reverses the stimulatory effects of activin (17-24). Follistatin, by interfering with the action of the endogenously produced activin, may decrease the expression of FSH β subunit gene and thus reduce the FSH synthesis and secretion by the gonadotropes (22-24, 25-27). However, whether follistatin may also act through a mechanism separated from activin is to date unclear. Actions of follistatin have been reported in systems where there is no or little evidence of activin expression, suggesting that at least in some tissues follistatin may act independently from activin (28). The purpose of this study was to test whether follistatin can suppress FSH levels *in vivo* in primates whose FSH secretion has been stimulated by activin or by GnRH.

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MATERIALS AND METHODS Drug
Recombinant human activin A (12) stored in 0.15M NaCl, 0.05M tris pH 7.5, was diluted in normal saline before injections. Recombinant human follistatin (16) was synthetized in the laboratory of Dr. Nicholas Ling and was provided in a lyophilized state by the Contraceptive Development Branch of the National Institute of Child Health and Human Development and was solubilized in 0.1N glacial acetic acid (HOAc). Gonadotropin releasing hormone (Factrel, Ayerst, Lab. Inc. New York) was diluted in normal saline. 0.1N HOAc and saline were used for the vehicle injections.
Animals Six prepubertal long-term castrate male monkeys (*Macaca fascicularis)* were studied. The monkeys weighed 2.1-2.3 Kg and were 2.5-3 year old.
Protocol The 6 prepubertal male monkeys each received activin (90 μg/kg) plus vehicle or human recombinant follistatin at the dose of 90 μg/kg or 180 μg/kg. The same animals received in random order the same doses of follistatin plus GnRH at the dose of 10 μg/kg. At least one week separated one injection from another. Follistatin and activin or follistatin and GnRH were injected i.v. simultaneously. Each peptide was injected separately through the two lesser saphenous veins. Three of the six animals were injected with the activin/GnRH vehicle. Blood samples were drawn before and frequently after the injections for 12 hours. Bio FSH and LH serum levels were measured in those samples. All samples from the activin injected animals and GnRH injected animals were respectively measured in the same assay. All experiments were approved by the Animal Care Committee of the University of Washington and were performed in accord with the highest standards of humane animal care.
FSH bioassay: FSH serum levels were measured by a previously validated assay (29) with minor

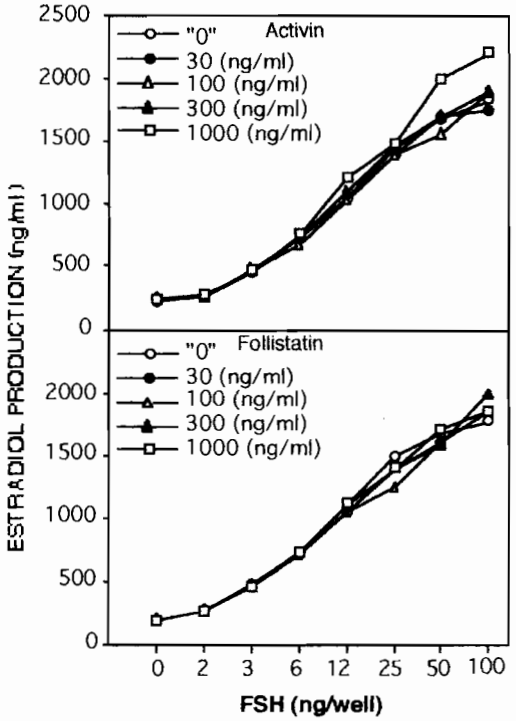

FIG 1 Estradiol production from granulosa cell cultures treated with increasing concentrations of human recombinant activin A (upper panel) and follistatin (lower panel) added together with the FSH standard. Data are presented as mean ± SEM of triplicate cultures.

modifications. Granulosa cells obtained from female Sprague Dawley DES-implanted rats were separated by collagenase, plated at 10,000 cells/well and cultured for 72h in serum free medium (total volume: 10 µl). Following this incubation, the medium was replaced with medium with standard or serum as previously described (29). Following a 72h incubation, the supernatant was collected for estradiol measurements by RIA. A urinary gonadotropin preparation (MAIACLONE; Serono; FSH biopotency 2286 IU/mg) was used as a standard reference preparation. Samples were analyzed in triplicate. The sensitivity of the FSH bioassay was 0.75 ng/well; the intra and inter-assay CVs were 10% and 14%, respectively. It has been reported that the presence of activin in the culture medium enhances the secretion of estradiol from granulosa cells in response to FSH (15, 24) Follistatin causes a dose-dependent decrease of the activin stimulatory effect on the granulosa cells but has no effect in the absence of activin (24,28). To determine whether the presence of activin or follistatin in our samples might affect the FSH bioassay, we added increasing activin and follistatin concentrations to the FSH standards. In the range of FSH concentrations that we detected in these animals (100-300 ng/ml), addition of either activin A or follistatin did not affect the estrogen production in our assay at any concentration (Fig 1). Methodological differences, such as the pre incubation of the cells with serum-free media and/or the cell concentration, may account for the lack of response to activin that we see in this assay in comparison with previous reports (15, 24, 28).

LH bioassay: Bioactive serum levels were measured by an *in vitro* MA-10 cell bioassay (30). The sensitivity of the assay was 0.05 mIU/well. The intra assay CV was 12%. Progesterone was measured using kit for Diagnostics System laboratories (Webster, Texas).

Statistics The existence of a statistical difference with time and between treatment was assessed by analysis of variance with repeated measures (ANOVA). $P<0.05$ was considered significant. The area under the response curve of the plasma hormone levels versus time (A.U.C.) was calculated according to the trapezoidal rule. Basal values were subtracted at each point before calculating the A.U.C.

RESULTS Activin The injection of activin significantly increased FSH serum bioactivity (A.U.C. vehicle: n=3, 7.3±18.2 ng/ml/hour; activin: n=6, 47.9±14.2 ng/ml/hour; $p<0.05$). Follistatin at both doses significantly suppressed the FSH increase caused by activin (A.U.C. activin plus: follistatin 90 µg= 29.2±9.7ng/ml/hour; follistatin 180 µg= 22.7±3.4 ng/ml/hour; $p<0.05$ vs. vehicle and activin) (fig 2). Mean baseline bio FSH levels did not differ between treatments (activin plus: vehicle= 132.2±16.2 ng/ml; follistatin 90 µg= 131.2±15.0 ng/ml; follistatin 180 µg= 128.1±19.8 ng/ml; p=N.S.). Activin had no detectable effect on LH bioactivity (data not shown). GnRH Following GnRH injection, FSH serum levels were significantly increased compared to basal levels (A.U.C. vehicle: n=3, 7.3±18.2 ng/ml/hour; GnRH 36.2±19.6 ng/ml/hour; $p<0.05$). Neither dose of follistatin affected the GnRH-stimulated FSH increase (A.U.C. GnRH plus: follistatin 90 µg= 50.7±6.9 ng/ml/hour; follistatin 180 µg= 56.9±8.6 ng/ml/hour; $p<0.05$)(fig 2). Follistatin did not affect the GnRH-induced increase of LH (data not shown).

DISCUSSION We administered human recombinant follistatin simultaneously with activin or with GnRH to prepubertal castrate male monkeys. We demonstrated that follistatin suppressed the activin-stimulated increase of FSH while it had no effect on the FSH increase stimulated by GnRH. These data provide the first evidence that follistatin can suppress FSH *in vivo* in a primate. The selective ability of follistatin to suppress the activin-stimulated FSH with no effect on the GnRH-stimulated FSH, suggests that this effect was due to neutralization of the activin action rather than a direct effect of follistatin on the pituitary.

We have demonstrated that activin is able to increase FSH in prepubertal castrate male monkeys as well as in adult monkeys (11, 15). In prepubertal

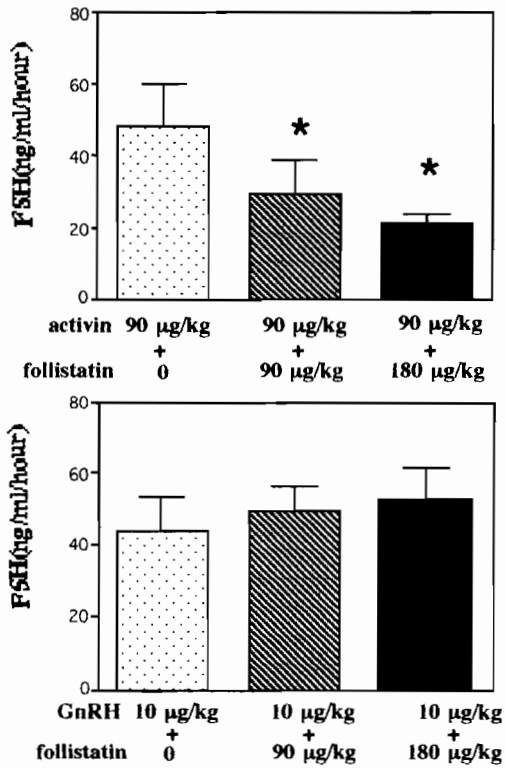

FIG 2 Average area under the curve of bio FSH serum levels following injections of activin (90 µg/kg) (upper panel) and GnRH (10 µg/kg) (lower panel) plus vehicle (⊡) or follistatin at the dose of 90 µg/kg (▨) or 180 µg/kg (■) in six prepubertal castrate male monkeys. Blood samples were taken frequently for 12 hours following each injection. * = $p<0.05$ vs. activin plus vehicle.

animals, human recombinant activin injected i.v. stimulated FSH secretion in a dose-dependent mannerand with a mechanism independent of GnRH (11). The ability of follistatin to selectively suppress the activin-stimulated FSH and not the GnRH-stimulated FSH further suggests that these two hormones act through different mechanisms. Our results are in agreement with *in vitro* and *in vivo* data reporting a suppressive effect of follistatin on FSH (7-9, 22-23). In pituitary cell cultures, purified follistatin decreased FSH synthesis and secretion (22-23) and in ovariectomized female rats the same preparation of human recombinant follistatin that we used in this study has been shown to be a potent suppressor of FSH (16). The mechanism by which follistatin suppresses FSH is not clear. Co-expression of mRNA and protein for activin and follistatin in the pituitary has suggested that follistatin might control FSH secretion through the paracrine/autocrine modulation of activin bioavailability (25,27,31).

In our study, the FSH-suppressing activity of follistatin is most likely due to neutralization of the activin effect. Follistatin injected at the same time as activin might have bound activin in the peripheral circulation and modified its clearance, or prevented its effect at the pituitary level by regulating the delivery or the binding to its own receptors. Our data are in contrast with data reported in pituitary cell cultures where follistatin could suppress FSH stimulated by either activin or GnRH (20, 23). The reason for this difference is unclear. However, since activin is locally produced within the pituitary, the possibility that in cultures follistatin may act through the binding of endogenous activin even in the presence of GnRH cannot be ruled out. On the other hand, it is also possible that higher doses of follistatin or a longer time of exposure might be necessary for follistatin to block GnRH effects on the pituitary *in vivo*.

Follistatin did not completely prevent the stimulation of FSH by activin and a FSH increase was still present following the injection of the highest dose of follistatin. The nature of activin-follistatin binding is still largely unknown. It is likely that the highest dose of follistatin that we used in this study was not sufficient to bind and therefore neutralize all the activin that we injected.

In conclusion our results indicate that human recombinant follistatin can suppress FSH *in vivo* in the non-human primate and that this effect may be due to neutralization of the activin effect on the pituitary. Further studies are necessary to determine the mechanism(s) by which follistatin modulates the activin effects.

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