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Effects of Castration and Recombinant Human Inhibin Administration on Circulating Levels of Inhibin and Gonadotropins in Adult Male Monkeys

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ABSTRACT: Inhibin has been suggested to play a role in gonadal feedback regulation of follicle-stimulating hormone (FSH) secretion; however, neither the half-life nor the time course of action of recombinant inhibin has been reported in any primate species. We sought to determine the disappearance half-life of circulating endogenous inhibin following castration in adult male monkeys, *Macaca fascicularis,* and to determine the half-life of administered recombinant human inhibin A and its effect on bioactive FSH and luteinizing hormone (LH) levels in castrate monkeys. Endogenous inhibin fell from 8,122 ± 2,077 U/L (mean ± SEM, $n = 5$) prior to castration to 383 ± 84 U/L at 24 hours and 269 ± 44 U/L at day 21 ($P < 0.05$ at 24 hours vs. day 21) (detection limit of assay 234 U/L). The early phase half-life of endogenous inhibin was 34 minutes (between 8 and 60 minutes) and a later phase half-life of 75 minutes was observed between 1 and 4 hours following castration. Recombinant inhibin exhibited a 14-minute early phase half-life between 8 and 60 minutes following the 5 μg intravenous (IV) recombinant inhibin dose, and a later phase half-life of 70 minutes between 1 and 4 hours in castrate monkeys ($n = 3$). Serum inhibin levels were maintained within or above the precastration range for 15 minutes. Single dose recombinant inhibin, 100 μg subcutaneous (SC) or intramuscular (IM) administered to castrate monkeys ($n = 3$), achieved and maintained normal serum inhibin levels for 6 hours. When bioactive FSH and LH responses were assessed by either the posttreatment nadir value or the area under the curve of the posttreatment values relative to the pretreatment baseline, no significant effect was observed following the 0-, 0.5-, 5-, or 50-μg IV recombinant inhibin dosages over the 10-hour sampling period. We conclude first that virtually all circulating inhibin is produced by the testis in male monkeys. Second, recombinant inhibin has a short early phase half-life in monkeys, similar to that of endogenous inhibin. Third, inhibin's putative effect of suppressing FSH secretion may require more prolonged replacement in long-term castrate monkeys. Fourth, IM or SC administration or recombinant inhibin appears to be a suitable means of achieving replacement serum inhibin levels in castrate monkeys.

Key words: FSH, LH, *Macaca fascicularis,* testis.

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Inhibin was originally proposed as a nonsteroidal gonadal regulator of pituitary gonadotropin secretion (McCullagh, 1932). The demonstration of follicle-stimulating hormone (FSH)-suppressing activity in steroid-free follicular fluid and testicular extracts supported this hypothesis and led to the eventual isolation and purification of inhibin, a glycoprotein consisting of one α and one β subunit linked by disulfide bonds (Ling et al, 1985; Miyamoto et al, 1985; Rivier et al, 1985; Robertson et al, 1985). Subsequent cloning of the inhibin α and β subunit genes and expression in mammalian cell culture systems has led to the availability of recombinant human inhibin for *in vitro* and *in vivo* studies (Mason et al, 1985; Forage et al, 1986).

The testis is a major source of circulating inhibin in several species; however, inhibin subunit genes are also expressed in several extragonadal tissues, where inhibins or activins may have important paracrine functions (Meunier et al, 1988). Using an assay directed against the α subunit of inhibin, Abeyawardene et al (1989) observed that levels rose through puberty in the monkey and levels decreased more than 80% following castration in adults, although low levels remained detectable by this assay. Keeping et al (1990) reported that inhibin levels in castrate adult monkey serum were below the detectability limit of an assay directed against purified bovine inhibin. Thus, the testis appears to be the major, but perhaps not exclu-

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sive, source of endogenous inhibin. The half-life of circulating endogenous inhibin appears to be relatively short, based upon an initial half-life of disappearance of inhibin of approximately 28 minutes following castration in a single juvenile male monkey supported by exogenously administered gonadotropin releasing hormone (GnRH) (Winters et al, 1991). The time course of clearance of endogenous inhibin and administered recombinant inhibin has not been reported in adult monkeys.

Purified and recombinant inhibin preparations have FSH-suppressing activity in nonprimate species (Findlay et al, 1987; Ying et al, 1987; Carroll et al, 1991; DePaolo et al, 1991; Rivier et al, 1991a,b; Robertson et al, 1991). Several lines of evidence also suggest a role for inhibin in the regulation of FSH secretion in primates. Studies in monkeys have shown FSH suppression by the administration of crude sources of inhibin (charcoal-extracted follicular fluid) and FSH stimulation by immunoneutralization of endogenous inhibin with specific inhibin antisera (Channing et al, 1981; diZerega et al, 1981; Rettori et al, 1982; Schenken et al, 1984; Abeyawardene and Plant, 1989; Medhamurthy et al, 1990, 1991). However, the effects of recombinant inhibin have not been reported previously in any primate species.

In this study of adult male monkeys, we determined the source of endogenous inhibin by measuring inhibin disappearance following castration. We demonstrated the time course of disappearance of single doses of recombinant human inhibin administered by intravenous (IV), subcutaneous (SC), and intramuscular (IM) routes to castrate animals. We also examined the effects on circulating levels of FSH and luteinizing hormone (LH) of various dosages of recombinant inhibin administered by single IV injection to adult male monkeys several weeks following castration.

Materials and Methods

Animals

Adult male macaques, *Macaca fascicularis* (weighing 4.2–6.2 kg), were housed under controlled conditions of light (on at 0600 hours; off at 1800 hours), temperature, and humidity in individual cages in the Regional Primate Research Center at the University of Washington. In addition to *ad libitum* monkey chow and tap water, the animals received fresh fruit, chewable vitamins, and iron injections. These studies were conducted in accordance with the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Surgeries

All surgeries were performed under aseptic conditions on animals anesthetized with fluothane. To facilitate blood sampling from unsedated and unrestrained animals, venous catheters were placed 4 days prior to study. Each animal was fitted with an indwelling femoral venous catheter (silastic tubing, OD 0.76 mm), which was tunneled SC from the inguinal region and exteriorized distally through a midscapular skin incision. The animals were jacketed, and the external portion of the catheter was protected by a flexible stainless steel housing attached to a sampling port mounted on the top of the animal's cage. Patency of the catheters was maintained by an infusion of 200 ml/day of heparinized (3,000 USP units/L) normal saline.

Drugs

The synthesis and purification of this recombinant human inhibin A preparation has been previously described (Tierney et al, 1990). Briefly, the preparation consists of two isoforms, 31K and 34K, made up of either a 21K or a 24K α subunit joined with the 15K β subunit. The apparent size difference is probably due to differences in glycosylation of the α subunit. Recombinant inhibin was lyophilized with bovine serum albumin (BSA) (5 mg BSA/mg inhibin) to facilitate solubility in aqueous solution. The inhibin was redissolved and diluted in normal saline with 0.5% BSA as 1-ml aliquots of 0.5, 5, and 50 μg/ml. Aliquots were stored at −20°C and thawed immediately before use.

Hormone Assays

Serum Inhibin—Serum inhibin was measured by heterologous double antibody radioimmunoassay (RIA), which uses an antiserum to purified 31K bovine follicular fluid inhibin and 31K bovine inhibin labeled with ^{125}I as tracer. The assay standard is pooled serum from women undergoing ovarian stimulation for *in vitro* fertilization. This assay has <1% cross-reactivity with the related polypeptides bovine activin A, bovine mullerian inhibitory substance, and porcine and human transforming growth factor-β, as well as free α and β inhibin subunits obtained by reduction and alkylation of 31K inhibin. Cross-reactivity does occur with the α-derived peptide, pro α-C; however, it is unknown whether this precursor peptide circulates in primate serum. The sensitivity of the assay at a sample volume of 100 μl was 234 U/L and the intraassay coefficient of variation was 10.7%. This assay has been validated previously for the measurement of inhibin in two monkey species, including *M. fascicularis* (Fingsheidt et al, 1989). We found that intact monkey serum diluted serially in castrate monkey serum and the addition of various amounts of recombinant inhibin to serum from castrate monkeys resulted in parallel displacement of tracer in the assay (data not shown).

Serum FSH and LH Bioassays—Bioactive FSH and LH levels in serum were measured by *in vitro* granulosa cell (Dahl et al, 1989) and Leydig cell (Steiner et al, 1980) bioassays, respectively. The standard reference preparation for both bioassays was a human pituitary gonadotropin preparation (LER-907; FSH biopotency 20 IU/μg; LH biopotency, 60 IU/mg by the second international reference preparation (IRP) of human menopausal gonadotropin standard). The sensitivities of the FSH and LH bioassays were 1.5 ng/well and 0.15 ng/well, respectively. the intraassay coefficients of variation for the FSH and LH bioassays for castrate monkey serum samples were 21% and 18%, respectively. We assessed whether recombinant inhibin in serum samples would affect performance of each bioassay prior to the study. The addition of 500 ng/ml recombinant human inhibin (equiv-

alent to an inhibin concentration of 1,500,000 U/L) to the assay standards had no effect upon estrogen production by granulosa cells in the FSH bioassay. The added inhibin minimally decreased testosterone production in the Leydig cell bioassay but only at very low levels of LH standard. Inhibin was not added to tissue culture wells for either bioassay during the subsequent gonadotropin measurements in this study.

Experimental Design

Study 1: Site of Origin and Clearance Pattern of Endogenous Inhibin—Five animals underwent blood sampling (1 ml) immediately before and at 1, 3, 7, and 21 days following castration. Indwelling femoral venous catheters were placed immediately prior to castration in three additional animals for more frequent sampling of endogenous inhibin levels in the early postcastration period. Blood samples were obtained in these latter animals immediately prior to castration and at 4, 8, 15, 30, and 60 minutes, and 2, 4, 8, and 24 hours following castration.

Study 2: Inhibin Patterns in Serum of Castrate Monkeys Following IV, IM, and SC Administration of Recombinant Inhibin—Following the completion of the above protocol, three animals received a single IV dose of 5 μg recombinant inhibin followed by blood sampling to determine the time course of disappearance of the recombinant inhibin. To avoid inhibin contamination of the sampling catheter, the recombinant inhibin was administered via the saphenous vein of the leg opposite the femoral catheter, following sedation with 50 mg ketamine IV. Blood samples (1 ml) were obtained immediately prior to each inhibin injection and at 4, 8, 15, 30, and 60 minutes, and 2, 4, 8, and 24 hours following the injection. In three additional animals, 100 μg of recombinant inhibin dissolved in 0.25 ml normal saline with 0.5% BSA was administered IM and SC on consecutive days beginning 2 days following castration. Blood samples were obtained in the unanesthetized animals through the indwelling venous catheter immediately prior to the inhibin injection and at 4, 8, 15, 30, and 60 minutes; and 2, 3, 4, 6, 8, 12, and 24 hours following each inhibin injection.

Study 3: Dose–Response Effects of Recombinant Inhibin on Bioactive LH and FSH Levels—Three to 4 weeks following castration, five animals received each of four doses of recombinant inhibin (0, 0.5, 5, and 50 μg in 1 ml 0.5% BSA) in random order by slow (~1-minute) IV injection through the indwelling venous catheter. Each dose was separated by a 3–4-day washout period. Blood samples (1 ml) were obtained in the unanesthetized animals through the indwelling venous catheter at 30-minute intervals for 2.5 hours prior to each inhibin injection and then at hourly intervals for the next 10 hours following the inhibin injection. Sampling began at the same time on each of the study dates (0700–0800 hours).

Blood samples were allowed to clot at 4°C. Serum was then collected and stored at −20°C until assay. All samples from an individual animal were run in the same LH and FSH bioassay to avoid the effects of interassay variability.

Statistical Analysis

The disappearance half-life of endogenous and recombinant inhibin was determined by use of nonlinear least-square analysis (RSTRIP, MicroMath Scientific Software, Salt Lake City, Utah) of the time–concentration curves generated from frequent sampling in each animal. Because inhibin levels approached the limit of assay detectability beyond 4 hours following either castration or the administration of recombinant inhibin, disappearance half-life was determined with values obtained only in the first 4 hours after each manipulation. The change in FSH and LH levels following each IV dosage of inhibin was assessed for each animal by analysis of variance with repeated measures, comparing the percent change for the lowest FSH and LH value relative to the mean baseline value preceding each dosage. The area under the curve for the levels of each gonadotropin during the 10 hours of sampling following each dosage was also compared by analysis of variance with repeated measures.

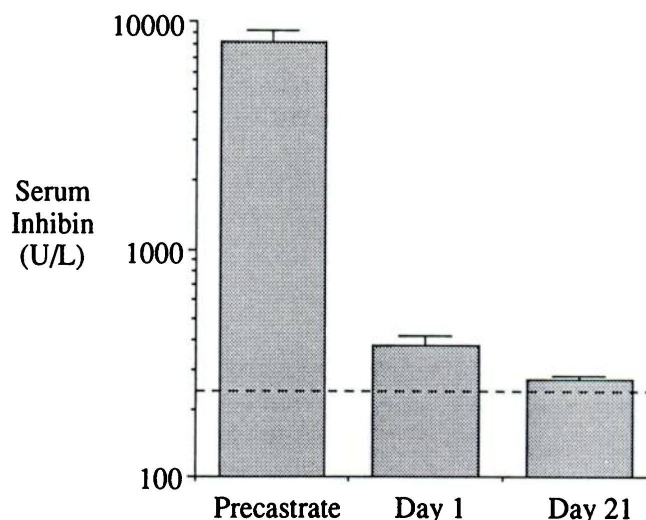

FIG. 1. Effect of castration on serum inhibin levels (mean ± SEM) in adult male monkeys (n = 5). Serum inhibin levels are expressed on a log scale with the lower limit of assay sensitivity indicated by the dashed line. Inhibin levels decreased by more than 95% in the first 24 hours and approached the lower limit of assay sensitivity by day 21 following castration.

Results

Study 1: Site of Origin and Clearance Pattern of Endogenous Inhibin

Endogenous inhibin levels decreased by more than 95% in the first 24 hours following castration, from 8,122 ± 2,077 U/L (mean ± SEM, n = 5) just prior to castration to 383 ± 84 U/L at 24 hours (Fig. 1). By 21 days following castration, inhibin levels declined further to 269 ± 44 U/L, which was very near the lower limit of assay detectability. In more detailed studies, inhibin levels were monitored frequently over the first 24 hours following castration (Fig. 2). A slight initial postcastration rise in serum inhibin was observed in each animal, presumably due to manipulation of the testes. Because of this initial inhibin rise, the disappearance half-life was calculated by using inhibin values beginning 8 minutes following cas-

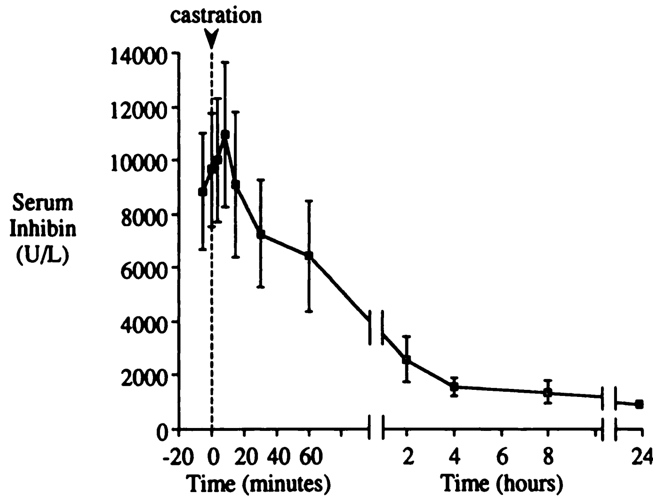

FIG. 2. Serum inhibin levels (mean ± SEM) in the first 24 hours following castration (denoted by dashed line at time 0) in adult male monkeys ($n = 3$).

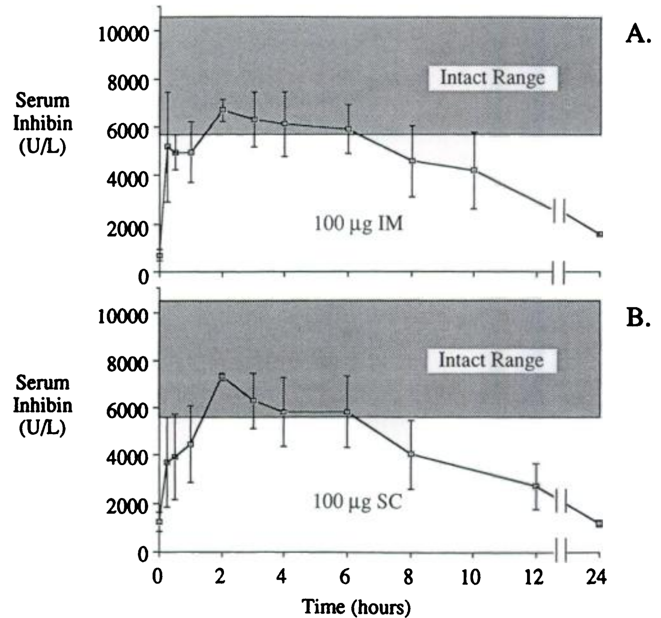

FIG. 4. Serum inhibin levels (mean ± SEM) following 100 µg recombinant inhibin administered IM (A) and SC (B) to castrate adult male monkeys ($n = 3$) at time 0. The range of inhibin levels for intact animals is illustrated by the shaded area for comparison.

tration. At least two components to the inhibin disappearance were observed with an early phase half-life of 34 minutes (between 8 and 60 minutes following castration) and a later phase half-life of 75 minutes (between 1 and 4 hours following castration).

Study 2: Inhibin Patterns in Serum Following IV, IM, and SC Administration of Recombinant Inhibin

Following the 5-µg IV dose of recombinant inhibin, serum inhibin levels rose from below the limits of assay detectability in the long-term castrate animals ($n = 3$) to a peak of 14,685 ± 2,011 U/L at 1 minute and fell rapidly thereafter (Fig. 3). The initial disappearance half-life of the administered recombinant inhibin was 14 minutes. Similar to the disappearance curve of endogenous inhibin, the fall in serum inhibin levels between 1 and 4 hours following recombinant inhibin administration demonstrated a half-life of 70 minutes. Inhibin levels were maintained within or above the precastration range for about 15 minutes following the 5-µg IV dose.

Inhibin levels rose to within the range of intact animals within 1–2 hours following IM administration of the 100-µg recombinant inhibin dose. These levels were maintained within the normal range for 6 hours. Similar results were obtained following SC administration of the 100-µg dose to the same animals (Fig. 4).

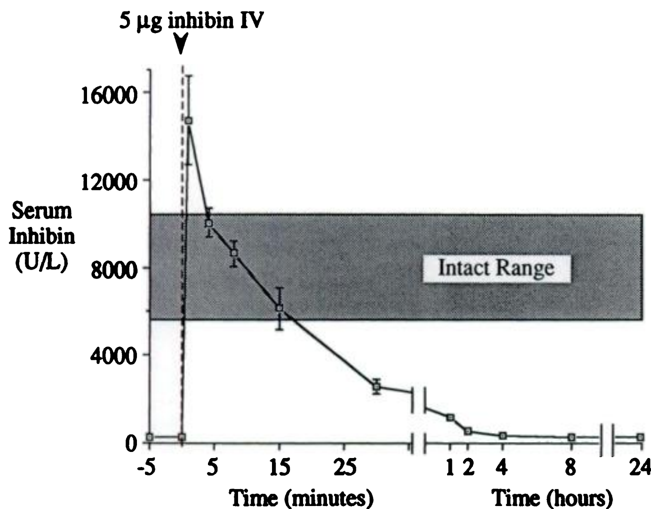

FIG. 3. Serum inhibin levels (mean ± SEM) following IV administration of 5 µg recombinant inhibin to long-term castrate adult male monkeys ($n = 3$) at time 0. The range of inhibin levels in intact monkeys is illustrated by the shaded area for comparison. Serum inhibin levels remained at or above the intact range for 15 minutes.

Study 3: Dose–Response Effects of Recombinant Inhibin or Bioactive LH and FSH Levels

The effect of inhibin administration on serum bioactive FSH and LH was analyzed for each animal by measuring the area under the curve of the FSH and LH levels over the 10 hours following each IV recombinant inhibin dosage and comparing this value to that generated by placebo administration in that animal. Additionally, the lowest FSH and LH value following each dose relative to the mean preinjection baseline (from five samples over 2.5 hours prior to inhibin injection) for each sampling period was compared to the lowest relative FSH and LH value following placebo. Although there was a trend toward decreased FSH levels following the 50-µg IV recombinant inhibin dose (Fig. 5), the effect was not statistically sig-

nificant. No significant effect of IV recombinant inhibin was observed on FSH or LH levels in the castrate monkeys following any of the other dosages tested.

Discussion

The rapid and profound decrease in serum inhibin levels following castration in this study demonstrates that circulating immunoreactive inhibin originates almost exclusively from the testis in this primate species. The initial half-time of disappearance was 34 minutes, with an 80% decrease within 4 hours and a 95% decrease within 24 hours. By 21 days following castration, serum inhibin levels were near or below the limit of assay detectability and were consistently undetectable at later time points (basal levels for IV recombinant inhibin administration experiments). Similar to the results of this study, circulating inhibin is cleared rapidly following castration in humans and rats with an early phase half-life of several minutes (Robertson et al, 1988; Ishida et al, 1990). In an earlier study performed on a juvenile monkey supported by exogenously administered GnRH, the early phase half-life of disappearance of endogenous inhibin following castration was 28 minutes (Winters et al, 1991), similar to the 34-minute half-life reported for adult monkeys in this study. Inhibin subunit mRNA and protein expression has been demonstrated in the testes of rats and primates (Roberts et al, 1989; Keeping et al, 1990), but inhibin subunit gene expression has also been demonstrated in several extragonadal tissues where inhibin may serve a paracrine role (Meunier et al, 1988). The castration studies indicate that these extragonadal sources of inhibin do not contribute significantly to circulating blood levels of inhibin.

The pattern of clearance from the circulation of recombinant inhibin administered IV is generally similar to that of endogenous inhibin. The initial disappearance of recombinant inhibin was more rapid than that observed for endogenous inhibin, 14 versus 34 minutes, respectively. This difference may be due to the effects of continued initial distribution of the recombinant inhibin in the first several minutes following its administration. Because of the transient rise in inhibin levels observed immediately after castration (presumably an artifact due to manipulation of the testes), the initial disappearance half-life of endogenous inhibin was calculated using values beginning 8 minutes following castration, possibly masking a more rapid early phase disappearance. However, with the same time points for the recombinant inhibin disappearance, the calculations suggest an initial recombinant inhibin disappearance half-life of 18 minutes. This apparent discrepancy may have several explanations. First, it is conceivable that administered inhibin may be more rapidly metabolized in the long-term castrate animal. Second, the

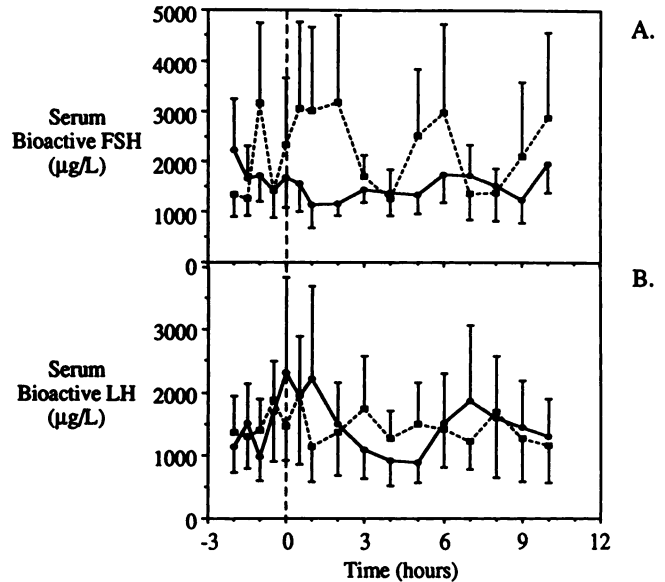

FIG. 5. Effect on serum bioactive FSH (mean ± SEM) (A) and bioactive LH (mean ± SEM) (B) of 50 μg recombinant inhibin (solid circles, solid lines) versus placebo (open squares, dashed lines) administered IV at time 0 to long-term castrate adult male monkeys (n = 5). No significant effect on bioactive FSH or LH was observed with this or smaller dosages of inhibin.

testis may be producing additional longer half-life forms of inhibin or related products detected by the assay that are not in the recombinant preparation. Due to this rapid clearance, inhibin levels were maintained within the precastrate range for a mean of only 15 minutes following the 5-μg recombinant inhibin dose. Rapid clearance of recombinant inhibin when administered IV may limit the effectiveness of inhibin administration by this route at suppressing gonadotropin secretion.

Substantial evidence exists in support of the hypothesis that inhibin plays a role in the gonadal feedback suppression of FSH secretion. Suppression of serum FSH and pituitary FSH β mRNA levels has been observed within several hours of the administration of recombinant inhibin in several studies in rats (Carroll et al, 1991; DePaolo et al, 1991; Rivier et al, 1991a,b; Robertson et al, 1991). Charcoal-extracted porcine follicular fluid suppresses FSH in male and female juvenile monkeys as well as intact and long-term castrate adult female monkeys (Channing et al, 1981; diZerega et al, 1981; Rettori et al, 1982; Schenken et al, 1984). In hypothalamus-lesioned adult male rhesus monkeys receiving an invariant pulsatile GnRH drive, testosterone replacement alone at the time of castration failed to prevent postcastration hypersecretion of FSH, whereas combined testosterone and charcoal-extracted porcine follicular fluid replacement maintained circulating FSH at concentrations similar to those observed prior to castration (Abeyawardene and Plant, 1989). Immunoneutralization of circulating inhibin in hypothalamus-

lesioned juvenile and adult male rhesus monkeys receiving an invariant pulsatile GnRH drive results in a selective hypersecretion of FSH (Medhamurthy et al, 1990, 1991). These immunoneutralization studies provide good evidence in support of a physiological role of inhibin in the gonadal feedback suppression of FSH secretion.

Although we did not demonstrate clear suppression of FSH levels by inhibin administration in this study, an important physiological role for inhibin in gonadotropin control cannot be excluded. First, the dosages of IV inhibin employed in this study may have been insufficient to suppress FSH, given the very brief increases in inhibin levels. The largest dose employed was 50 μg or approximately 10 μg/kg. Although suppression of FSH by IV recombinant inhibin at a dose of 4 μg/kg was observed in male rats in one study (Rivier et al, 1991a), inhibin levels achieved were not reported, making comparisons with our dosage difficult. Most such studies in rats have employed larger relative single dosages of inhibin administered by IV or SC routes (Tierney et al, 1990; Carroll et al, 1991; Robertson et al, 1991).

It is also possible that the long-term castrate monkeys used in this study may be less sensitive to the feedback effects of inhibin. Long-term castrate monkeys are relatively resistant to the feedback effects of gonadal steroids (Resko et al, 1977; Plant et al, 1978); a similar phenomenon may exist for nonsteroidal regulators of gonadotropin secretion. Such a phenomenon has been observed in studies of FSH suppression by bovine follicular fluid administration in castrate female rats (deJong et al, 1979). We chose a long-term castrate model in our initial study for several reasons. First, we considered the possibility that in the intact state, feedback effects of endogenous inhibin and gonadal steroids may limit further suppression of FSH by exogenous inhibin. Previous monkey studies suggest that the feedback effects of testosterone may partially mask the suppression of FSH by inhibin (Medhamurthy et al, 1991). We also sought to achieve a state of elevated FSH levels against which suppressive effects of various dosages of inhibin might be observed. Although mean baseline FSH levels during the 2 weeks of inhibin administration and washout did not increase significantly over time, the substantial short-term spontaneous fluctuations of bioactive FSH levels characteristic of the castrate animal may have obscured a small effect of inhibin. However, we suspect that an insufficient dose or duration of administered recombinant inhibin is the likely reason for the failure to observe FSH suppression in this model. It will be necessary to examine more prolonged administration of recombinant inhibin to explore inhibin's potential further in the control of FSH secretion in primates.

We explored other possible routes of administration of recombinant inhibin as a practical way to achieve inhibin levels that remain in the precastrate range. Our single dose study of 100 μg of recombinant inhibin by IM and SC routes achieved such inhibin levels for several hours. Prolonged administration of inhibin by these routes may be a practical way of maintaining inhibin levels within the normal range for future examination of the role of inhibin in regulating gonadotropin secretion.

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