

# Inhibin-B: A Likely Candidate for the Physiologically Important Form of Inhibin in Men

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## ABSTRACT

Inhibin is a glycoprotein hormone that is defined on the basis of inhibition of pituitary FSH production. However, previous data have not shown any correlation between RIA measurements of inhibin and FSH in men.

New enzyme-linked immunosorbent assays, specific for inhibin A, inhibin B, and inhibin pro- $\alpha$ C-related immunoreactivity, were applied to the measurement of inhibin in 32 healthy men. Further measurements of inhibin B and pro- $\alpha$ C-RI were carried out on groups of men exhibiting a wide range of FSH concentrations, including semen donors, infertile men, and men with elevated FSH concentrations.

Inhibin A was undetectable (<2 pg/mL) in all men studied. The

healthy men studied all had measurable concentrations of inhibin B (135.6 pg/mL; confidence interval, 108.4–169.4) and pro- $\alpha$ C-RI (426.3 pg/mL; confidence interval, 378.4–480.2). A close negative correlation was found between the inhibin B and FSH concentrations in the semen donors ( $r = -0.69$ ;  $P < 0.001$ ), the infertile men ( $r = -0.81$ ;  $P < 0.001$ ), and the men with elevated FSH concentrations ( $r = -0.54$ ;  $P < 0.01$ ), but not in a group of healthy volunteers ( $r = -0.08$ ;  $P = \text{NS}$ ). No correlation was observed between concentrations of pro- $\alpha$ C-RI and FSH in any of the groups studied.

These results strongly suggest that the physiologically important form of inhibin in men is inhibin B, which has a critical effect on FSH release. Inhibin B may offer a clinically useful serum marker of testicular function. (*J Clin Endocrinol Metab* 81: 1321–1325, 1996)

**I**NHIBIN HAS been defined as a glycoprotein hormone of gonadal origin, consisting of two dissimilar disulfide-linked subunits ( $\alpha$  and either  $\beta$ A or  $\beta$ B), that has an inhibitory effect on pituitary gonadotropin production (1). The inhibin  $\alpha$ - and  $\beta$ -subunits are synthesized as proproteins (pro- $\alpha$ N- $\alpha$ C and pro- $\beta$ - $\beta$ ; Fig. 1) (2). Inhibin is secreted into biological fluids in a variety of molecular forms, including inhibin precursors, although the rate at which the different molecular forms are synthesized remains uncertain (3). Inhibin bioactivity is dependent on formation of the  $\alpha/\beta$  dimer, whereas the circulating free forms of the  $\alpha$ -subunit have no suppressive effect on pituitary FSH secretion (4).

There has long been considerable skepticism concerning the importance of inhibin in the regulation of FSH secretion in men. This has been due to the absence of any clear evidence for a functional relationship between circulating inhibin and FSH levels in men with reproductive disorders (5). The specific measurement of dimeric inhibin in the circulation, however, is problematic. Most of the existing studies on circulating inhibin concentrations in men are based on a heterologous assay (6), which is now known not to distin-

guish between the dimeric (active) form and free (biologically inactive)  $\alpha$ -subunit (4, 7). The inhibin forms detectable by this assay are present in the circulation of healthy men at concentrations that are increased by stimulation with gonadotropins, particularly FSH (8–11). However, with the exception of hypogonadotropic conditions (11), there was no evident difference in the concentrations of these inhibin forms between fertile and infertile men. In particular, no inverse relationship was observed between the measured inhibin concentration and FSH (5), thus raising the possibility that the inhibin forms measured with this assay do not reflect the level of bioactive hormone present in men.

Recently, a new assay format has been developed (12) that has been adapted to the specific measurement of different circulating inhibin forms. This format has been adapted to provide assays capable of specifically measuring the bioactive inhibin dimers, inhibin A and inhibin B (13, 14). In addition, a third inhibin enzyme-linked immunosorbent assay has been developed that measures the concentration of the inhibin  $\alpha$ -subunit precursor pro- $\alpha$ C but is also known to have a potential for cross-reaction with other inhibin forms containing the precursor pro sequence (15). Immunoreactivity measured in this assay is, therefore, termed pro- $\alpha$ C-related immunoreactivity (pro- $\alpha$ C-RI).

Using these assays, we have shown that serial changes can be observed in the peripheral concentrations of dimeric inhibin during the menstrual cycle of women (13, 14) and that the different inhibin forms exhibit different patterns of se-

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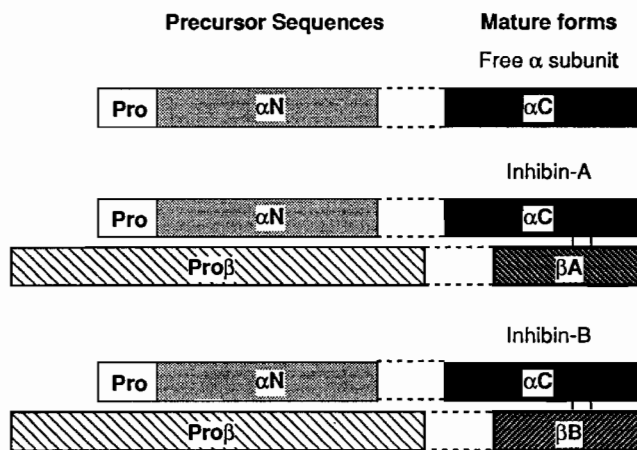


FIG. 1. Diagrammatic representation of the different inhibin forms, free  $\alpha$ -subunit (lacking inhibin bioactivity), inhibin A, and inhibin B, as well as the precursor sequences. Inhibin is known to be present in biological fluids in a wide variety of combinations of these sequences.

cretion, with inhibin A being predominantly secreted by the dominant follicle and corpus luteum, whereas, in contrast, inhibin B rises early in the follicular phase, then falls thereafter (14).

In this report, we have applied these assays to measurement of the circulating inhibin forms in different groups of men, including healthy volunteers, semen donors, infertile men with normal and abnormal sperm production, and men with elevated FSH concentrations.

### Experimental Subjects

#### Groups 1a and 1b (healthy volunteers)

Inhibin concentrations were measured in a total of 32 normal men. The subjects in group 1a ( $n = 16$ ) were between 23–50 yr of age and healthy, with no apparent reproductive disorders. They were taking no medication and were all of proven fertility. All had plasma concentrations of testosterone, LH, and FSH in the normal range. A further subset (group 1b) of male volunteers, aged 19–45 yr, participating in contraceptive trials was also studied. Inclusion criteria for this group included a normal medical history and physical examination; the absence of current use of prescription medications; normal basal serum LH, FSH, and T levels; at least three successive normal seminal fluid analyses (sperm count,  $>20$  million/mL; oval forms,  $>50\%$ ; motility,  $>50\%$  after 48-h abstinence) on specimens collected at 2-week intervals, and normal values on routine hematology, blood chemistry, urinalysis, and fasting lipid profile. Exclusion criteria included any history of significant acute or chronic medical illness, alcohol abuse, anabolic steroid abuse, or reproductive dysfunction. The inhibin data were pooled for these two subgroups of normal volunteers, but as FSH measurements in the two subgroups were carried out by different assay methodologies, the correlation calculations were carried out separately.

#### Group 2: semen donors

A group of 18 healthy males (aged 24–28 yr) who were donors in a university-based therapeutic donor insemination program were selected on the basis of the absence of any heritable disease, the absence of any sexually transmitted disease, and several semen samples yielding at least 100 million motile sperm/ejaculate.

#### Group 3: men with infertility

A group of 30 men from couples presenting with infertility of at least 2-yr duration were studied. These men ranged from 28–46 yr of age, were healthy, and had two or more semen analyses during their infertility workup. These semen analyses were defined by WHO (16) criteria

as normal ( $n = 6$ ), abnormal ( $n = 21$ ), or showing complete azoospermia ( $n = 3$ ).

#### Group 4: men with elevated FSH concentrations

A group of men (aged 20–45 yr) with primary testicular disease of varying causes and severity leading to elevated basal FSH levels was studied. The types of testicular disease included undescended testes, previous chemotherapy, idiopathic oligo/azoospermia, and testicular trauma. These men had no other known disease, alcohol use, or anabolic steroid use.

## Materials and Methods

### The inhibin assays

The inhibin A, inhibin B, and pro- $\alpha$ C-RI assays are two-site enzyme-linked immunoassays based on the use of plates coated with specific capture antibodies to the inhibin  $\beta$ A- and  $\beta$ B-subunits and the  $\alpha$ -subunit pro sequence, respectively. The F(ab) fraction of a mouse monoclonal antibody (R1) to the N-terminal portion of the 20-kDa inhibin  $\alpha$ -subunit conjugated to alkaline phosphatase was used for detection in all three assays. Coefficients of variation were less than 5% within a plate and less than 7% between plates for all three assays.

### Inhibin A

Inhibin A assays were carried out as previously described (13). Activin A, activin B, follistatin, purified human pro- $\alpha$ C, and inhibin B all had less than 0.1% cross-reaction. Assay sensitivity was 2 pg/mL.

### Inhibin B

The inhibin B assay used a capture antibody raised against a sequence from near the C-terminal of the human  $\beta$ B-subunit, as previously described (14). The monoclonal antibody was immobilized on a hydrazide plate as described for the inhibin A assay. Before assay, all samples were treated for 3 min at 100 C to eliminate nonspecific binding, then pretreated with 1% hydrogen peroxide for 30 min. Both male and female patient samples diluted out in parallel to the recombinant inhibin B standard. Activin A, activin B, follistatin, and purified human pro- $\alpha$ C all had less than 0.1% cross-reaction, whereas inhibin A had 0.5% cross-reaction in the inhibin B enzyme-linked immunosorbent assay. The assay detection limit was 5 pg/mL. The recovery of added recombinant inhibin B to postmenopausal serum samples was quantitative (115%).

### Pro- $\alpha$ C-RI

The pro- $\alpha$ C-RI assay has recently been described in detail (15). The assay used a capture monoclonal antibody raised against the inhibin  $\alpha$ -subunit pro sequence. Both male and female patient samples could be diluted out in parallel to the purified ( $>75\%$  purity) pro- $\alpha$ C preparation that was used as standard. Recombinant forms of activin A, activin B, inhibin A, inhibin B, and follistatin all had less than 0.02% cross-reaction. The sensitivity of this assay was 3 pg/mL. This assay may not be completely specific for pro- $\alpha$ C, as previously described immunoblotting experiments (15) indicated a potential for cross-reaction with larger forms of dimeric inhibin containing the  $\alpha$ -subunit pro sequence, such as pro- $\alpha$ N $\alpha$ C/ $\beta$ A, pro- $\alpha$ N $\alpha$ C-pro- $\beta$ / $\beta$ A, pro- $\alpha$ N $\alpha$ C/ $\beta$ B, and pro- $\alpha$ N $\alpha$ C-pro- $\beta$ / $\beta$ B (Fig. 1). However, the lack of suitably purified preparations of these inhibin forms makes it impossible at present to quantify the degree of cross-reactivity in the pro- $\alpha$ C-RI assay.

### Other assays

To investigate the applicability of the results to different clinical situations, the inhibin measurements were correlated with gonadotropin estimations obtained using three different assay systems in the three centers. The concentrations of FSH and LH were measured for the subjects in group 1a by previously described RIAs (17) with assay sensitivities of 0.9 and 0.8 IU/L, respectively, and within-assay variabilities of 5.0% and 4.6%, respectively. Testosterone was measured by previously described RIA (18). For subjects in groups 2 and 3, FSH and LH were measured using a chemiluminescent assay (Imulite, Diagnostic

Products Corp., Los Angeles, CA). Interassay variation for FSH was 8.1%, and intraassay variation was 5.4%. The LH assay had an interassay variation of 6.5 and an intraassay variation of 6.1%. For subjects in groups 1b and 4, FSH and LH were measured by the Delfia fluorimunoassay (Wallac Oy, Turku, Finland). The sensitivities of the Delfia assays were less than 0.016 and less than 0.019 IU/L for FSH and LH, respectively. Intraassay coefficients of variation were 5.9% for FSH and 4.5% for LH. Interassay coefficients of variation were 9.3% for FSH and 7.4% for LH. Testosterone was assayed by RIA using reagents from the WHO matched reagent program. The assay sensitivity was 0.35 nmol/L; the inter- and intraassay variabilities were 8.1% and 4.1%, respectively.

### Statistical analysis

All immunoassay hormone values are assumed to have a log normal distribution. The average values shown, therefore, are geometric means  $\pm$  95% confidence intervals. The relationship between FSH and other hormones was investigated by the use of correlation coefficients for log-transformed data, which were tested for significance by calculation of the *t* statistic.

Informed consent was obtained from all subjects. Ethical approval for the above studies was obtained from the University of Texas Southwestern Medical Center ethics of medicine committee and the University of Washington human subjects committee.

## Results

### Concentrations of inhibin forms in normal men

The inhibin measurements from the two groups of healthy volunteers (1a and 1b) were pooled. Inhibin A was undetectable ( $<2$  pg/mL) in all healthy men studied, but all had measurable concentrations of inhibin B [135.6 pg/mL; confidence interval (CI), 108.4–169.4] and pro- $\alpha$ C-RI (426.3 pg/mL; CI, 378.4–480.2).

### Relationship between inhibin forms and FSH

The relationships between individual inhibin measurements and gonadotropin measurements by chemiluminescent assay are shown in Fig. 2 for the subjects in group 2 (semen donors) and group 3 (men with infertility). The semen donors had a mean inhibin B concentration of 178.12 pg/mL (CI, 140.56–225.72) and a mean pro- $\alpha$ C-RI concentration of 518.0 pg/mL (CI, 415.5–645.9), whereas the infertile men had a mean inhibin B concentration of 66.02 pg/mL (CI, 45.76–95.26) and a mean pro- $\alpha$ C-RI concentration of 354 pg/mL (CI, 297.7–417.2). Significant correlations were found between the serum FSH and inhibin B concentrations in both the semen donors ( $r = -0.69$ ;  $P < 0.001$ ) and the infertile men ( $r = -0.81$ ;  $P < 0.001$ ). No significant correlation was found between the plasma concentrations of FSH and testosterone in either the semen donors ( $r = -0.11$ ;  $P = \text{NS}$ ) or the infertile men ( $r = -0.23$ ;  $P = \text{NS}$ ). No correlation was found between the plasma concentrations of FSH and pro- $\alpha$ C-RI in either the semen donors ( $r = -0.10$ ;  $P = \text{NS}$ ) or the group of infertile men ( $r = -0.16$ ;  $P = \text{NS}$ ). The inhibin B concentration did not correlate significantly with the LH concentration in donors, but there was a significant correlation in the infertile men ( $r = -0.47$ ;  $P < 0.05$ ). No significant correlation was observed between the concentrations of inhibin B and pro- $\alpha$ C-RI in either the semen donors or the infertile men.

The relationship between individual inhibin measurements and gonadotropin measurements by fluorimunoassay are shown in Fig. 3 for the subjects in group 1b (healthy volunteers) and group 4 (men with elevated FSH concentrations).

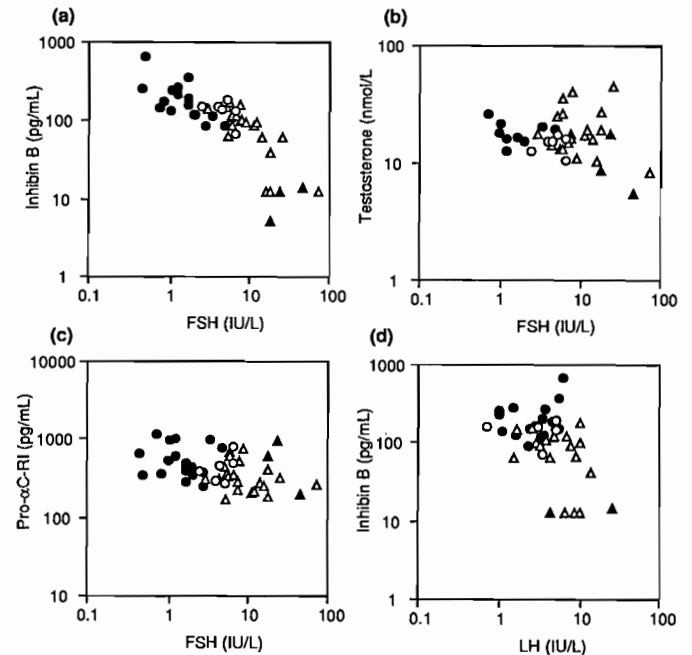


FIG. 2. Individual concentrations of inhibin B (a), pro- $\alpha$ C-like immunoreactivity (b), and testosterone (c) plotted against FSH for healthy semen donors (●) and infertile men with normal semen analysis (○), abnormal semen analysis (△), and complete azoospermia (▲). d, Individual concentrations of inhibin B plotted against LH for the same subjects.

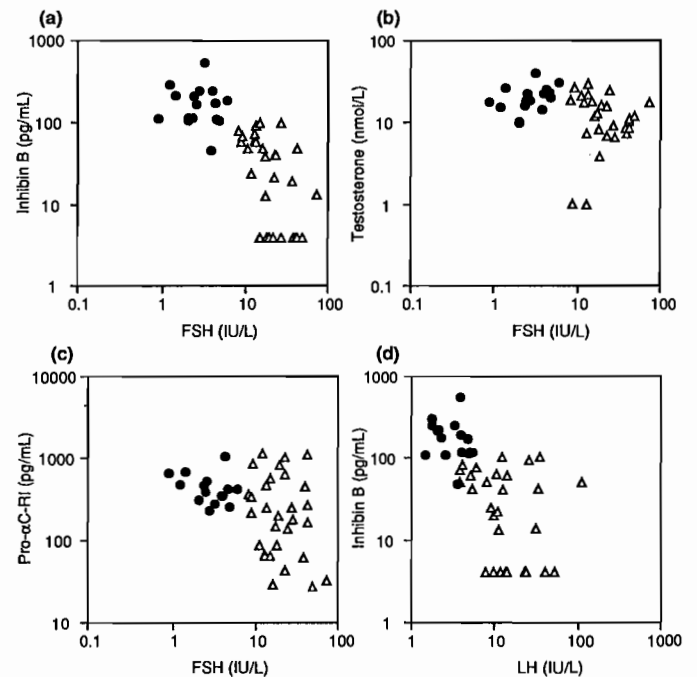


FIG. 3. Individual concentrations of inhibin B (a), pro- $\alpha$ C-like immunoreactivity (b), and testosterone (c) plotted against FSH for healthy volunteers (●) and men with elevated FSH concentrations (△). d, Individual concentrations of inhibin B plotted against LH for the same subjects.

The men with elevated FSH concentrations had a mean inhibin B concentration of 21.08 pg/mL (CI, 13.64–33.54) and a mean pro- $\alpha$ C-RI concentration of 191.2 pg/mL

(CI, 126.0–290.2). A significant correlation was found between the serum FSH and inhibin B concentrations in the men with elevated FSH concentrations ( $r = -0.54$ ;  $P < 0.01$ ), although no significant correlation between inhibin B and FSH was seen in the healthy volunteers ( $r = -0.08$ ;  $P = \text{NS}$ ). No significant correlation was found between FSH and either testosterone or pro- $\alpha$ C-RI in either the healthy volunteers or the men with elevated FSH concentrations. No significant correlation was observed between the concentrations of inhibin B and pro- $\alpha$ C-RI in either the semen donors or the infertile men.

### Discussion

This is the first report of an inverse relationship between inhibin and FSH in the circulation in men with both normal and abnormal spermatogenesis. These data offer the strongest evidence presented to date of a critical role for inhibin in the regulation of gonadotropin secretion in men. To investigate the applicability of this observation, we tested it in a variety of clinical conditions involving comparisons with three different FSH assays. A weak inverse correlation was also observed between the circulating concentrations of inhibin B and LH, although this was restricted to men with abnormal spermatogenesis or FSH concentrations and was not observed in either healthy men or semen donors. The mechanism for this is unknown. Our finding that inhibin A is undetectable in men has previously been reported (19), but this is the first report to demonstrate that inhibin B is the predominant form of bioactive inhibin in men. We recently demonstrated that in women, inhibin A and inhibin B circulate in very different patterns throughout the menstrual cycle (14). Inhibin B is the first hormone released by the ovary in response to the intercycle rise in FSH, whereas inhibin A is a later product of the mature preovulatory follicle and the corpus luteum.

The undetectable level of inhibin B found in azoospermic men who are otherwise healthy suggests that the source of inhibin B is probably the testis, although no data are yet available concerning inhibin B concentrations after orchidectomy. The inhibin subunits are known to be synthesized in the testis, as has been demonstrated by immunohistochemical localization of the  $\alpha$ - and  $\beta$ A-proteins (20) and the  $\alpha$ - and  $\beta$ B-subunit messenger ribonucleic acids (mRNAs) by *in situ* hybridization (21). Although mRNA for the inhibin subunits has been demonstrated in other anatomical sites (22), notably the adrenal gland (23), inhibin immunoreactivity has been found to be higher in testicular veins than in peripheral veins (24, 25) and disappears after castration (24). The apparent absence of inhibin A from the circulation of adult males indicates that if present at all within the testis, this substance must have a purely paracrine role (26).

We previously reported that men and women have similar concentrations of pro- $\alpha$ C-related immunoreactivity (15). This assay measures the biologically inactive inhibin  $\alpha$ -subunit precursor pro- $\alpha$ C, but will also detect precursor forms of the biologically active inhibin dimers. Nonetheless, the wide discrepancy between the concentrations of pro- $\alpha$ C-RI and the bioactive inhibin dimers as well as the absence of any relationship between the concentrations of FSH and pro-

$\alpha$ C-RI suggest that measurements made by the pro- $\alpha$ C-RI assay predominantly reflect the concentration of biologically inactive  $\alpha$ -subunit precursors. The similarity in physiological patterns seen between this assay and inhibin measurements made by the original Monash assay (5) as well as the close correlation previously observed between individual samples in the female menstrual cycle (15) suggest that these two assays are probably detecting similar inhibin forms.

The inhibin concentration was higher in men with apparently normal fertility than in those with infertility and abnormal spermatogenesis. Indeed the highest concentrations were seen in the young group of selected fertile semen donors. Immunoreactive inhibin is known to originate primarily from the Sertoli cells. It is possible that the circulating inhibin B concentrations reflect the extent of the interaction between Sertoli cells and germ cells (27), particularly the elongate spermatids (28). It is notable that all men in this study with azoospermia and consequently no germ cell/Sertoli cell interaction had barely detectable concentrations of inhibin B. The inhibin B concentration in azoospermic men with normal FSH concentrations is unknown. The wide range of circulating pro- $\alpha$ C-RI concentrations in these men and the absence of any correlation with inhibin B suggest that the  $\alpha$ -subunit may be under independent hormonal control and that the level of  $\beta$ B-subunit expression determines the rate of synthesis of bioactive inhibin B in the testis. This is supported by the observation that testicular stimulation with hCG and human menopausal gonadotropin results in an increase in mRNA for inhibin  $\alpha$ -subunit, but no change in  $\beta$ B-subunit mRNA (21).

At present, there is widespread concern about the possibility of an increasing level of fertility impairment in men resulting from adverse environmental or toxicological damage to testicular function (29). Although major degrees of cytotoxic damage to the testis will cause a reduction in the overall inhibin concentration (30), it is not known whether minor degrees of damage to Sertoli cells or germ cells result in any change in the inhibin B concentration. However, the apparently constitutive nature of inhibin B synthesis and the inverse relationship with FSH raise the possibility that inhibin B may represent a clinically useful marker of Sertoli and/or germ cell function. Such a marker would prove to be an invaluable tool for both clinical assessment of the individual patient as well as future clinical and toxicological studies of male fertility.

In conclusion, these data suggest that, contrary to current suggestions (31), there may be a consistent relationship between inhibin levels and reproductive function in men. There is thus support for the original hypothesis, advanced many years ago (32), that inhibin of testicular origin may be important in the regulation of pituitary FSH secretion in the adult male.

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