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Physiological relationships between inhibin B, follicle stimulating hormone secretion and spermatogenesis in normal men and response to gonadotrophin suppression by exogenous testosterone

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Inhibin has been postulated to be secreted by Sertoli cells in response to follicle stimulating hormone (FSH) and in turn to exert an inhibitory effect on FSH production. We have investigated this relationship using an assay specific for dimeric inhibin B. A total of 56 normal men received 200 mg testosterone enanthate (TE) i.m. weekly, for 65 ± 1 weeks in a trial of hormonal male contraception. Before treatment a significant negative correlation between inhibin B and FSH concentration ($r = 0.49$, $P < 0.001$) was observed. During TE treatment, luteinizing hormone (LH) and FSH were rapidly suppressed. This was followed by a parallel decline in inhibin B and sperm concentration. During the early recovery phase, inhibin B concentrations remained suppressed in men who showed a delay in resumption of spermatogenesis, despite higher FSH concentrations. Inhibin B returned to pretreatment concentrations after 24 weeks recovery, when the inverse relationship with FSH was restored. Our results showed the expected inverse physiological relationship between inhibin B and FSH in normal men, with a decline during TE treatment and α subsequent resumption of the inverse relationship during recovery. These data clearly support the hypothesis that inhibin B plays a physiological role in the feedback control of FSH secretion, and reflects FSH-stimulated Sertoli cell function.

Key words: contraception/FSH/inhibin B/male reproduction/spermatogenesis

Introduction

Inhibin is a glycoprotein of gonadal origin with inhibitory effects on gonadotrophin secretion, consisting of two subunits, α with either β_A or β_B (Burger and Igarashi, 1988). Many molecular forms are found in the circulation, although biological activity resides only in dimeric forms, inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$). A physiological function for a non-steroidal secretory product of the testis selectively to suppress pituitary FSH secretion has long been proposed (McCullagh, 1932), but evidence that inhibin fulfils this role has been inconsistent. A clear relationship between inhibin and follicle stimulating hormone (FSH) was not found in men with various reproductive disorders (de Kretser *et al*., 1989). Other studies have shown changes in inhibin concentrations in normal and hypogonadotrophic men consistent with stimulation of inhibin secretion by FSH. Thus administration of gonadotrophin releasing hormone (GnRH) increased inhibin concentrations in hypogonadotrophic men (Sheckter *et al*., 1988), and FSH increased inhibin concentrations in normal men following suppression of endogenous FSH by human chorionic gonadotrophin (HCG) administration (McLachlan *et al*., 1988). Androgen- or GnRH antagonist-induced gonadotrophin suppression resulted in a fall in inhibin concentrations (Bagatell *et al*., 1989; Wallace *et al*., 1993). Annual variations in inhibin concentrations have also been reported, increasing with luteinizing hormone (LH), FSH and testosterone in June with a nadir in August (Meriggiola *et al*., 1996).

Results from these studies were all obtained using a heterologous radioimmunoassay, which detects the biologically inactive free α subunit as well as dimeric inhibins (Schneyer *et al*., 1990). New immunoassays which specifically detect only dimeric inhibins with negligible cross-reactivity with the free α-subunit have recently been developed (Groome and O'Brien, 1994), allowing reinvestigation of the roles of inhibin in the control of FSH secretion and as a circulating marker of Sertoli cell function. Using these novel specific assays, it has recently been demonstrated that, unlike in the female, inhibin A is undetectable in the plasma of normal men, and that there is an inverse relationship between plasma concentrations of inhibin B and FSH in normal men and in pathological conditions (Anawalt *et al*., 1996; Illingworth *et al*., 1996; Nachtigall *et al*., 1996). Inhibin B has therefore been suggested to be the physiologically important form of inhibin in men.

We here report investigations into the physiological relationships between circulating inhibin B, FSH and spermatogenesis in normal men, and changes during exogenous administration of testosterone enanthate (TE) to suppress gonadotrophins and thus spermatogenesis. TE has recently been used in trials sponsored by the World Health Organization (WHO) as a prototype hormonal male contraceptive (WHO Task Force for the Regulation of Male Fertility, 1990; WHO Task Force on Methods for the Regulation of Male Fertility, 1996). Of interest is the consistent finding that suppression of spermatogenesis is not complete in all men, with ~30% maintaining a low rate of sperm production despite undetectable gonadotrophin secretion (WHO Task Force on Methods for the Regulation of Male Fertility, 1995; Handelsman *et al*., 1995). The basis for

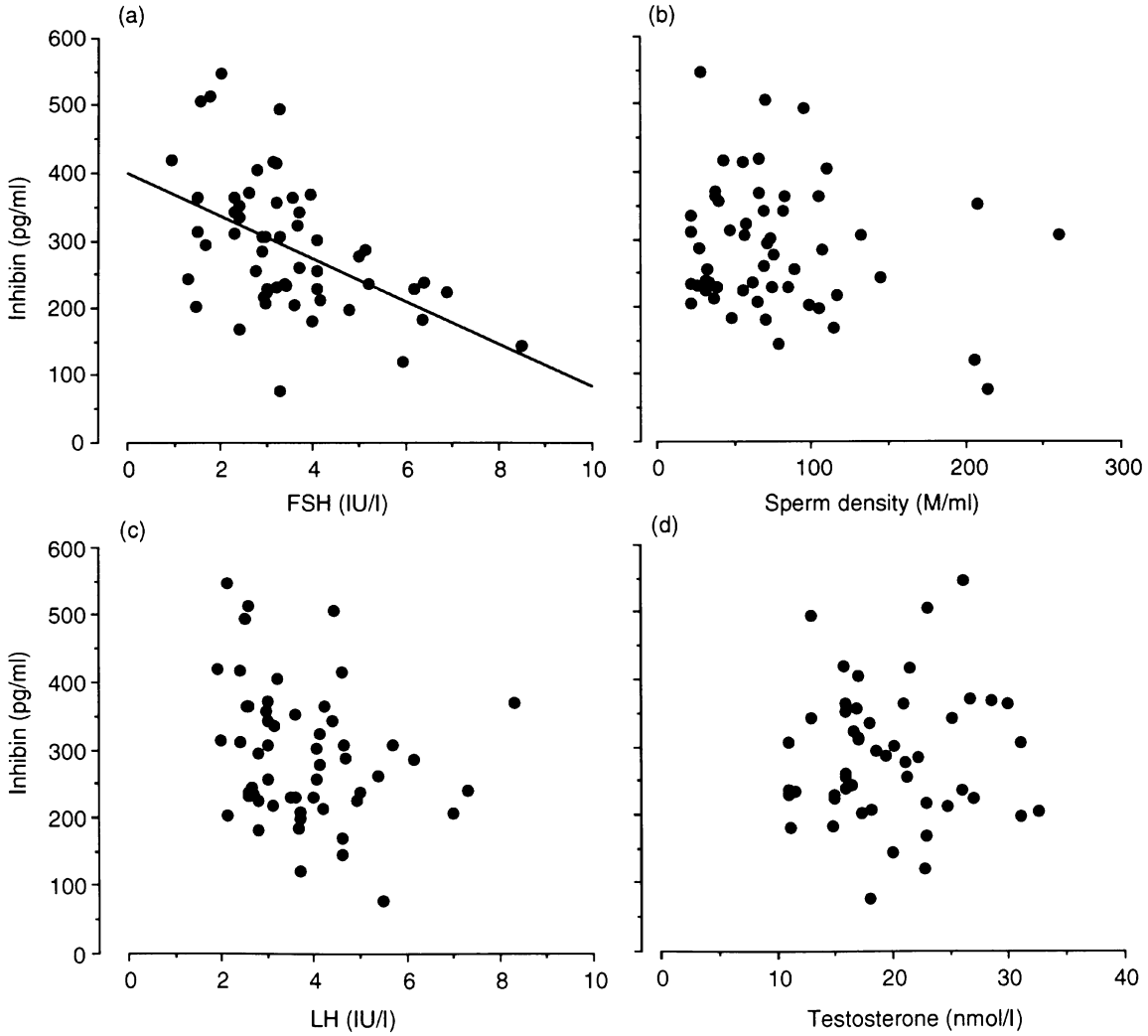

Figure 1. Individual concentrations of inhibin B in normal men pretreatment plotted against (**a**) follicle stimulating hormone (FSH) concentration, (**b**) sperm concentration, (**c**) luteinizing hormone (LH) concentration, and (**d**) testosterone concentration ($n = 56$).

this heterogeneity is unknown, although differences in androgen metabolism (Anderson *et al.*, 1996), and higher pretreatment and recovery FSH concentrations (Handelsman *et al.*, 1995) have been observed in azoospermic compared to oligozoospermic responders. There is currently no information on the state of the seminiferous epithelium or Sertoli cell function during or following suppression of spermatogenesis by TE, although inhibin B concentrations have been reported to be suppressed in normal men during treatment with combined TE and levonorgestrel (Anawalt *et al.*, 1996). Since circulating inhibin is a Sertoli cell secretory product (Steinberger and Steinberger, 1976; Roberts *et al.*, 1989; Vligen *et al.*, 1993), we have investigated the possibility that differences in the degree of suppression of spermatogenesis by TE are reflected in circulating concentrations of inhibin B.

Materials and methods

A total of 56 healthy caucasian men aged 21–41 years (mean 31 ± 2 SEM) were recruited to a multicentre clinical trial of hormonal male contraception (WHO Task Force on Methods for the Regulation of Male Fertility, 1996) after routine medical examination and biochemical screening and haematological analyses. On two occasions before TE treatment was started, all subjects were confirmed to have sperm densities of $>20\times10^6$/ml, and plasma concentrations of gonadotrophins and testosterone within the normal range. Subjects were administered 200 mg TE (Testoviron; Schering AG, Berlin, Germany) i.m. weekly, and were required to use this as their only method of contraception for 12 months once their sperm concentration had fallen to $<5\times10^6$/ml. The duration of treatment varied between individuals, as suppression of spermatogenesis to the required oligozoospermic threshold took between 10 and 26 weeks. The mean duration of treatment was 65 ± 1 weeks (range 53–80). Following the final TE injection, subjects were monitored until sperm concentration had returned to pretreatment values.

Semen samples were analysed according to WHO (1987) criteria after 3 days of ejaculatory abstinence at 2–4 week intervals throughout the study. Azoospermia was confirmed by examination of the pellet after centrifugation.

Blood samples were obtained prior to the first injection, after 12 weeks TE treatment, and on two occasions later, after 8–10 months and after 11–15 months of continued treatment. The recovery phase was defined as starting 1 week after the final TE injection, and blood and semen samples were taken after 12 and 24 weeks recovery. In α subgroup of 30 men additional blood samples were obtained 1, 2, 4,

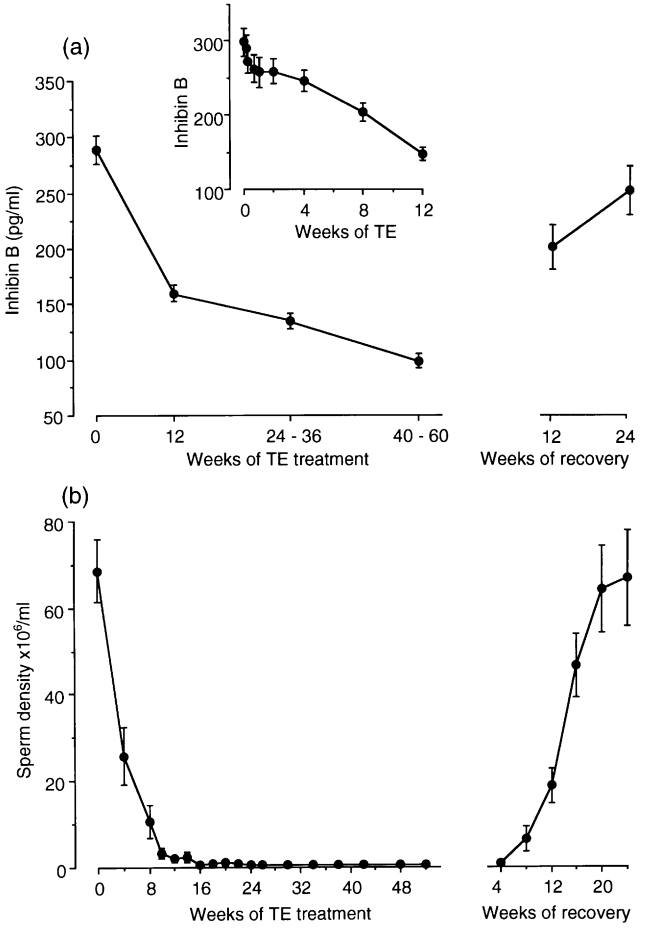

Figure 2. (**a**) Inhibin B concentration and (**b**) sperm concentration pretreatment, during testosterone enanthate (TE) treatment, and during the recovery phase. The inset shows changes in inhibin B concentration in the subgroup of 30 men over the first 12 weeks of TE treatment. Values are mean ± SEM (n = 56).

and 7 days after the first injection, and after 2, 4, and 8 weeks TE treatment. Blood samples were also obtained from this group after 4 and 8 weeks of the recovery phase.

Inhibin B assay

The assay is a two-site enzyme-linked immunoassay, using plates coated with antibody to the β_B subunit, and a second antibody directed against the *N*-terminal portion of the α subunit, conjugated to alkaline phosphatase, as previously described (Groome *et al.*, 1996). The monoclonal antibody was immobilized on a hydrazide plate, and prior to assay all samples were treated at 100°C to eliminate non-specific binding then treated with 2% hydrogen peroxide for 30 min. The assay has <0.1% cross-reactivity with activin forms, and <0.5% with inhibin A. Assay sensitivity was 10 pg/ml. Inter- and intra-plate coefficients of variation were <7 and <5% respectively.

Other assays

Plasma testosterone was measured by previously-described radio-immunoassay (Corker and Davidson, 1978). FSH was measured by time-resolved immunofluorescence (Delfia; Wallac, Turku, Finland) with an assay sensitivity of 0.06 IU/l. Intra- and inter-assay coefficients of variation were <8%.

Statistics

Values are expressed as mean ± SEM. Results were analysed by analysis of variance with Neuman–Keuls test for post-hoc analysis for repeated measures, or Student's *t*-test for single results. Correlation coefficients and statistical comparisons were performed on log-transformed data to correct for non-Gaussian distribution. The Mann–Whitney *U*-test was used for non-parametric data. Significance was determined at $P < 0.05$.

Results

Inhibin B was detectable in the plasma of all men pretreatment. The mean concentration of inhibin B in healthy normal subjects was 291 ± 13 pg/ml. There was a significant inverse correlation between inhibin B and FSH concentrations pre-treatment (r = –0.49, $P < 0.001$, Figure 1a). There was no correlation between inhibin B and sperm concentration (r = 0.19, ns, Figure 1b), LH concentration (r = 0.08, ns, Figure 1c) or testosterone concentration (r = 0.17, ns, Figure 1d) at that time, but there was a positive correlation between LH and testosterone pretreatment (r = 0.40, $P < 0.01$).

During administration of TE there was a fall in inhibin B concentrations ($P < 0.001$, Figure 2). Inhibin B concentrations fell rapidly over the first 12 weeks, then continued to fall more slowly during prolonged TE administration, reaching a nadir of 100 ± 6 pg/ml at the end of TE treatment. There was no relationship between duration of TE treatment and inhibin B concentration at the end of treatment. Following discontinuation of TE treatment, inhibin B concentration recovered to 202 ± 20 pg/ml at 3 months and 253 ± 21 pg/ml at 6 months, at which time it was not significantly different from pretreatment.

The more frequent blood sampling regimen of the subgroup allowed further investigation of the decline in inhibin B concentration at the beginning of TE treatment, and comparison with the rate of decline in sperm concentration. The fall in inhibin B concentration reached statistical significance only 4 days following the first injection (298 ± 18 pg/ml pretreatment, falling to 290 ± 19, 273 ± 17 and 263 ± 18 pg/ml after 1, 2, and 4 days, $P < 0.05$, Figure 2 inset). Sperm concentration fell in all men during TE treatment (Figure 2b), with a similar time course to the rapid phase of the decline in inhibin B concentration. Of the 30 men in the subgroup, 15 became azoospermic within 20 weeks of TE treatment at which time the sperm concentration in the remaining, oligozoospermic, responders was 2.0 ± 0.6×10^6/ml. There was, however, no difference in inhibin B concentrations pretreatment or during TE treatment between these two groups (Table I). Thus inhibin B concentrations continued to fall for the duration of TE treatment in those 15 men who rapidly achieved azoospermia, concentrations being at all time points similar to those who maintained a low rate of spermatogenesis. Sperm concentration continued to fall in the oligozoospermic responders, and by the end of TE treatment spermatozoa were detectable only in the centrifuged ejaculate of two men (concentration <0.1×10^6/ml).

To explore relationships between spermatogenesis and inhibin B and FSH concentrations more closely during the early recovery phase, α subgroup of 30 men were analysed in greater detail. Men were classified retrospectively by the rate of recovery of sperm concentration over the first 8 weeks of

Table I. Sperm concentration and inhibin B concentrations pretreatment and during testosterone enanthate (TE) treatment in azoospermic and oligozoospermic responders

	Weeks of TE treatment					
Sperm concentration ($\times 10^6$/ml)	Pretreatment	4	8	12	16	20
Azoospermic	62 ± 8	15 ± 8*	<0.1*	<0.1*	<0.1*	0*
Oligozoospermic	76 ± 13	39 ± 11	22 ± 7	3.5 ± 1.4	1.2 ± 0.5	2.0 ± 0.6
Inhibin B (pg/ml)	Pretreatment	1	2	4	8	12
Azoospermic	287 ± 29	270 ± 36	262 ± 30	261 ± 24	212 ± 19	150 ± 13
Oligozoospermic	296 ± 20	247 ± 18	256 ± 18	230 ± 15	195 ± 13	145 ± 10

Values are means ± SEM, $n = 30$. There was a significant (*$P < 0.001$) difference in sperm concentration between azoospermic versus oligozoospermic responders during TE treatment but not pretreatment, and no difference in inhibin B concentrations between the two groups at any time-point.

Table II. Sperm concentration, inhibin B and follicle stimulating hormone (FSH) concentrations pretreatment, at the end of testosterone enanthate treatment (EOT), and at 4 and 8 weeks into the recovery phase in normal men according to the speed of recovery of spermatogenesis

	Pretreatment	EOT	4 weeks	8 weeks
Sperm concentration ($\times 10^6$/ml)				
Group A ($n = 11$)	54 ± 6	0	0	0
Group B (n=19)	71 ± 11	0	1.5 ± 0.7	11 ± 5
Inhibin B (pg/ml)				
Group A	297 ± 34	85 ± 12	103 ± 14	90 ± 10
Group B	299 ± 21	105 ± 8	127 ± 10*	134 ± 12*
FSH (IU/l)				
Group A	3.7 ± 0.4	nd	4.9 ± 2.6	7.3 ± 1.2
Group B	2.9 ± 0.3	nd	2.4 ± 0.7**	4.6 ± 0.4**

Subjects were classified into groups A and B on the basis of sperm concentration after 8 weeks recovery phase: group A remained azoospermic at that time.
*$P < 0.05$, **$P < 0.005$ versus group A (analysis of variance).
nd = not detectable.

the recovery phase (Table II). Thus 11 of the 30 men remained azoospermic after 8 weeks (group A), at which time the mean sperm concentration in the other 19 men (group B) was 11 ± 5×10^6/ml. The duration of TE treatment did not differ between these two groups: 62 ± 2 weeks in group A versus 63 ± 3 weeks in group B. Group B showed a slower suppression of spermatogenesis, reaching a threshold of 5×10^6/ml after 9.2 ± 0.8 weeks compared to 5.2 ± 0.8 weeks in group A ($P = 0.001$). There were no differences in plasma inhibin B or FSH concentrations pretreatment or during TE treatment between these two groups, and although inhibin B levels at the end of TE treatment were lower in group A (85 ± 12 versus 105 ± 8 pg/ml), this did not reach statistical significance. However inhibin B concentrations did not then rise in group A, although there was a significant rise in group B, from 105 ± 8 to 134 ± 12 pg/ml ($P < 0.05$) after 8 weeks. Plasma FSH was suppressed to undetectable concentrations during TE treatment, but showed a 'rebound' during the recovery phase which was particularly marked in group A. Thus FSH concentrations were higher in group A compared to group B at both 4 and 8 weeks recovery: 4.9 ± 2.6 versus 2.4 ± 0.7 IU/l after 4 weeks, and 7.3 ± 1.2 versus 4.6 ± 0.4 IU/l after 8 weeks ($P < 0.005$).

After 24 weeks recovery, both inhibin B concentration and sperm concentration had recovered to pretreatment levels (Figure 2). FSH concentration was also similar to pretreatment (3.8 ± 0.3 IU/l pretreatment, 3.3 ± 0.3 IU/l after 24 weeks recovery). At this time, a significant inverse correlation between inhibin B concentration and FSH concentration was again found ($r = 0.45$, $P < 0.02$).

Discussion

Our results demonstrate a significant inverse relationship between plasma inhibin B and FSH in normal men under physiological conditions. This relationship was also found at the end of the recovery phase. A similar inverse correlation has been reported for a group of semen donors (Illingworth *et al*., 1996). These data therefore support the suggestion that inhibin B has a role in the physiological regulation of FSH secretion in men. No correlation between inhibin B and LH or testosterone was found, but there was a correlation between LH and testosterone concentrations before treatment. There was also no significant direct relationship between plasma inhibin B and sperm concentration before treatment. It has been suggested that inhibin secretion from Sertoli cells is regulated by interaction with germ cells (Pineau *et al*., 1990; Carreau, 1995), and expression of α- and β_B-subunit mRNA is maximal at stages of spermatogenesis which are maximally sensitive to FSH (Bhasin *et al*., 1989). Inhibin B concentrations are progressively lower in groups of men with increasing

spermatogenic damage, and are undetectable in men with azoospermia (Illingworth *et al*., 1996).

Inhibin B concentrations fell, as expected, during exogenous testosterone suppression of gonadotrophin secretion. FSH concentrations fall to ~25% after 7 days, and inhibition of gonadotrophin secretion is complete within 12 weeks of TE administration (Anderson and Wu, 1996). A statistically significant decline in inhibin B was detectable only 4 days after the first TE injection, and there was a rapid decline over the first 12 weeks of TE treatment. The time-courses of decline in inhibin B and sperm concentration were similar, with an initial rapid fall in both markers over the first 12 weeks followed by a second phase of continuing but slower decline, inhibin B concentrations eventually falling to 30% of pretreatment concentrations after over 1 year of treatment. A decline in inhibin B concentations in normal men during combined TE and levonorgestrel treatment has also been reported (Anawalt *et al*., 1996), the degree of decline in inhibin B being similar to that reported here, although dynamic changes during steroid suppression and the recovery phase were not demonstrated. It is thus possible to infer that ~70% of plasma inhibin B is gonadotrophin-dependent, which is in striking similarity to the finding that men with idiopathic hypogonadotrophic hypogonadism have inhibin B concentrations 25–30% of normal (Anawalt *et al*., 1996; Nachtigall *et al*., 1996). A significant proportion of plasma inhibin B (~25%) appears to be gonadotrophin-independent, which is only abolished in the presence of primary testicular disease, where inhibin B is undetectable (Illingworth *et al*., 1996). Inhibin is also secreted by Leydig cells (Risbridger *et al*., 1989), but it is unlikely that this would constitute a significant source in the absence of LH. The continuing slow decline in inhibin B concentration even after complete suppression of gonadotrophin secretion and achieving azoospermia may reflect a continuing change in the interaction between Sertoli cells and the germ cell population (Carreau, 1995).

During recovery from spermatogenic suppression on stopping TE, sperm concentration, inhibin B and FSH all increased towards the pretreatment range. The rates of recovery of these three markers, however, were different. While sperm concentration and inhibin B were still well below normal, after 4–8 weeks of recovery, FSH had already returned to the physiological range and indeed in some individuals showed a rebound rise to supraphysiological concentrations. Men showing a slower recovery of spermatogenesis had lower recovery-phase inhibin B concentrations, and a greater rise in FSH secretion. They also had a faster suppression of spermatogenesis, from a slightly but not significantly lower pretreatment sperm concentration. These results differ from findings using the heterologous inhibin assay, when plasma inhibin concentrations rapidly returned to normal in all men following withdrawal of TE treatment (Wallace *et al*., 1993). Similar results have been described during TE treatment (Handelsman *et al*., 1995) where men who became azoospermic showed a more rapid decline in spermatogenesis, had higher pretreatment FSH concentrations, and a rebound of FSH concentrations in the recovery phase. These changes and subtle differences between individuals during re-establishment of spermatogenesis and recovery from an experimentally induced hypogonadotrophic state clearly show the intimate relationship between FSH, inhibin B and sperm concentration. The FSH overshoot in the early recovery phase may be due to a relatively faster recovery of the gonadotrophs from TE suppression compared to the re-establishment of full Sertoli cell function and inhibin secretion leading to suboptimal negative feedback on FSH secretion. FSH returned to the normal pretreatment range later in recovery, as previously described (Handelsman *et al*., 1995), at which time the inverse relationship between FSH and inhibin B was re-established. Measurement of the specific dimeric form inhibin B therefore appears to allow the demonstration of physiological relationships underlying the previously described differences in FSH concentration, not explained by immunoreactive inhibin or testosterone concentrations. These data provide further evidence to support the postulated role of inhibin B in the hypothalamo–pituitary–testicular axis.

Incomplete suppression of spermatogenesis has been reported with all hormonal methods thus far tested, but the exact basis for this heterogeneity remains unclear. We have previously demonstrated differences in androgen metabolism between azoospermic and oligozoospermic responders (Anderson *et al*., 1996), which were apparent during TE treatment but not before treatment. Despite the differences in inhibin B during the recovery phase, inhibin B concentrations pretreatment and during TE treatment did not segregate the rapid from the slow responders. Measurement of plasma inhibin B does not therefore appear to be of value in predicting the spermatogenic response to sex steroid administration.

In conclusion, these results support the hypothesis that plasma inhibin B is involved in the physiological control of FSH secretion in men. Inhibin B concentrations were also correlated with the rate of recovery of spermatogenesis following prolonged suppression, at which time an inverse relationship to FSH was also found. Inhibin B is therefore an important additional marker of reproductive function in men.

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