

CLINICAL STUDY

Is inhibin B a pharmacodynamic parameter for FSH in normal men?

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Abstract

Objective: This study aims to investigate the pharmacodynamic effect of FSH on inhibin B serum levels in normal men in order to elucidate the physiological regulation of inhibin B secretion in more detail. **Design and Methods:** Injections of 3000 IU recombinant, human FSH (rhFSH) were followed by single-blinded injections of placebo, 1000 and 2000 IU rhFSH spaced by at least 28 days between injections. **Results:** After injection of 3000 IU rhFSH, inhibin B values were significantly elevated above baseline for 24, 96 and 120 h (maximal increase after 96 h, mean \pm S.E.M. 303 ± 18 pg/ml). Injection of 2000 IU rhFSH led to a significant increase in inhibin B (maximum mean \pm S.E.M. 318 ± 20 pg/ml) from 24 to 120 h. Injection of 1000 IU rhFSH led to a significant increase in inhibin B after 96 h (maximum mean \pm S.E.M. 300 ± 16 pg/ml). The inhibin B areas under the curve after injection of 2000 and 3000 IU rhFSH were significantly higher than those following the placebo and 1000 IU rhFSH. In the 12 fertile men investigated, at baseline a strong diurnal rhythm of inhibin B parallel to that of testosterone was observed. **Conclusions:** Serum inhibin B can be considered only a partial pharmacodynamic parameter of FSH *in vivo*, since the integrity of the spermatogenic process appears to be a second fundamental component in the regulation of its secretion from the testis.

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Introduction

The existence of inhibin as a suppressor of follicle-stimulating hormone (FSH) release has been postulated since the 1940s. Elucidation of the role of inhibin in the feedback system has been impeded by the lack of specific assays for the dimeric inhibin B that is secreted from the testis (1) and which is the physiologically important form of inhibin in males (2, 3). The development of new double antibody ELISAs for dimeric inhibins (4, 5) allowed the reinvestigation of inhibin physiology in males.

As a marker of spermatogenesis, inhibin B was shown to be low in patients with complete SCO (Sertoli cell only) and in patients with idiopathic hypogonadotropic hypogonadism when compared with normal controls (3, 6). In men with infertility, inhibin B levels are closely related to sperm concentration (7), total sperm count, testicular volume (8) and to the percentages of tubules with SCO and tubules with elongated spermatids (9).

In trials of male contraception, suppression of luteinizing hormone (LH) and FSH resulted in reduction of the inhibin B levels, which returned to normal in the recovery periods (3, 10). In men with isolated gonadotropin-releasing hormone (GnRH) deficiency, it

could be shown that inhibin B levels rise with increasing FSH but do not correlate with sperm density during further therapy (11). Moreover, in men with hematological malignancies, inhibin B levels fell significantly with increasing FSH values during chemotherapy (12), and in monkeys, it could be shown that inhibin B represents an early marker of testicular damage – one which is more sensitive than FSH (13).

Despite the strong negative correlation between FSH and inhibin B, and the known dependency of Sertoli cells on FSH stimulation (14), conflicting opinions about the site and regulation of inhibin B production exist (1). The relationship between FSH and inhibin B in the adult was investigated in 10 normal men who were injected with 3000 IU recombinant, human FSH (rhFSH) (3). This study showed a significant increase in serum inhibin B levels 1–7 days after FSH administration. However, no dose–response investigations had been performed. Results of chronic administration of FSH in infertile patients have shown conflicting results with unchanged (15) and increased (16) inhibin B plasma levels after 1–12 weeks of treatment. Therefore the question remained as to whether inhibin B is an FSH-dependent parameter of Sertoli cell function in normal men. The aim of this study was to estimate to what extent inhibin B is a pharmacodynamic parameter

for FSH action in men. This was estimated by using injections of a placebo, 1000, 2000 or 3000 IU rhFSH in normal men.

Subjects and methods

Subjects and study design

This study was performed in 12 healthy men, who were injected with FSH or a placebo. The work was approved by the Ethics Committee of the University and the State Medical Board, Münster. All volunteers gave written informed consent to participation in the study. Power analysis of the inhibin B levels of proven fathers (17) indicated that 10 normozoospermic men would be sufficient to detect an 80% difference between initial and stimulated inhibin values using a dose of 3000 IU rhFSH (as used previously by Anawalt *et al.* (3)). For the initial examination, 14 Caucasian fathers (time to pregnancy <12 months) aged 18–45 years were recruited by local press advertisement and were examined for normal general medical history, normal physical condition, normal blood values for routine clinical chemistry, hematology, normal reproductive hormones, normal semen parameters and normal sonography of scrotal content. Volunteers with clinically relevant abnormalities of the above-mentioned parameters were excluded; the remaining 12 fathers were rescreened for fulfillment of the inclusion criteria. The first injection (3000 IU) was followed by injections (injection volume 4 ml) of a placebo, 1000 and 2000 IU rhFSH, the injections being spaced by at least 28 days. All injections were given subcutaneously. The volunteers were blinded to the dose they received.

Following the injection of 3000 IU rhFSH, blood was sampled at 1, 2, 4, 10, 12, 14, 16, 18, 24, 36, 48, 60, 72, 84, 96, 120, 144, 192, 240, 288, 336 and 504 h. Hormone analysis after the first injection included determinations of FSH, LH, prolactin, inhibin B and testosterone. In addition, ultrasonography of scrotal content was performed 12, 48, 96, 144, 336 and 504 h after rhFSH injection. On the basis of these results, in the following trials blood was drawn at 24, 48, 72, 96 and 120 h after injection of rhFSH or placebo.

FSH was always injected in the morning between 0800 h and 1100 h. Venous blood was sampled between 0800 h and 1100 h at every visit. Blood samples for endocrine determinations were separated at 800 **g** and stored at -20°C until evaluation.

Assays

Consecutive samples for each volunteer were measured within one assay. Serum levels of LH, FSH and prolactin were determined by highly specific time-resolved fluoroimmunoassays (Autodelfia; Wallac, Turku, Finland). The lower detection limits for FSH

and LH were 0.25 IU/l and 0.12 IU/l respectively. The normal range in our laboratory for LH is 2–10 IU/l, that for FSH is 1–7 U/l and that for prolactin is <500 mU/l. The mean intra- and interassay coefficients of variation were 1.2 and 3.5% for LH, 1.3 and 4.7% for FSH, 1.0 and 5.3% for prolactin respectively.

Testosterone was determined by RIA (DSL-4100; Diagnostic Systems Laboratories, Sinsheim, Germany). The lower detection limit for testosterone was 0.17 nmol/l; the mean intra- and interassay coefficients of variation were 6.1 and 9.5% respectively. The normal serum level for testosterone is >12 nmol/l.

Inhibin B was measured using a commercially available, double antibody, enzyme-linked immunoassay (Serotec Ltd, Oxford, Oxon, UK). The lower detection limit for inhibin B was 7.8 pg/ml. In our laboratory, the normal range for serum inhibin B is 90–327 pg/ml, as obtained from 84 proven fathers. The mean intra- and interassay coefficients of variation for inhibin B were 3.3 and 19.0% respectively.

Serum levels of inhibin pro alpha C were analyzed at baseline and at 96 h after the injection of the placebo, 1000 and 3000 IU rhFSH. The inhibin pro alpha C subunit was measured using a commercially available, double antibody, enzyme-linked immunoassay (Serotec Ltd). The mean intra- and interassay coefficients of variation for pro alpha C were 4.0 and 4.2% respectively.

Screening clinical chemistry and hematology parameters were analyzed by using routine autoanalyzer methods (18).

Semen analysis

Semen samples were analyzed according to the World Health Organization Laboratory Manual (19) and subjected to rigid internal (20) and external quality control (21).

Ultrasonography of scrotal content

Sonographic (Sonoline Versa Pro; Siemens, Erlangen, Germany) measurements of testis volume were performed by applying a high-frequency 7.5 MHz sector scanner (22).

Statistics

All variables were checked for normal distribution in the Kolmogorov–Smirnov one-sample test for goodness-of-fit. Variations over time within one dose were evaluated by one-way ANOVA for repeated measurements. In the case of an overall $P < 0.05$ in the ANOVA, differences between baseline values and the following time points were tested by using Tukey's post hoc test. Variations between different doses and placebo injection were evaluated by two-way ANOVA for repeated measurements, followed by Tukey's post hoc

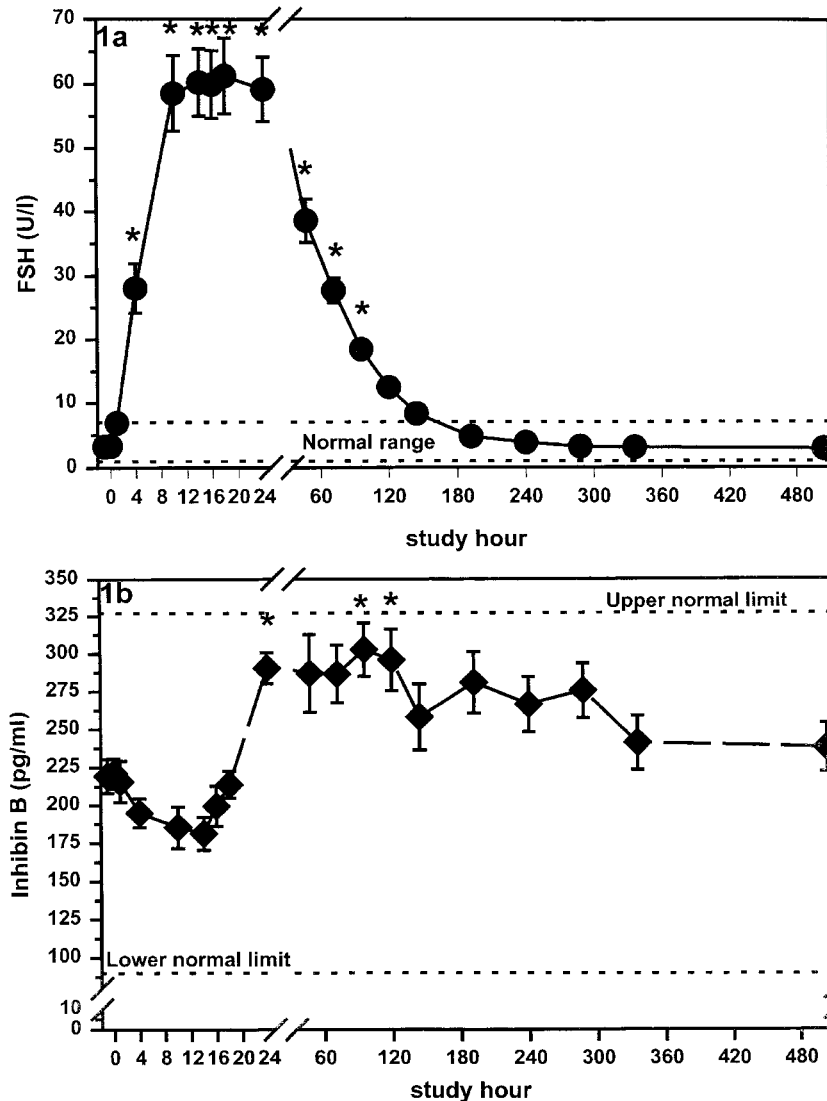


Figure 1 Serum FSH (a) and serum inhibin B levels (b) after injection of 3000 IU rhFSH. Significant differences relative to baseline values are indicated by asterisks. Results are presented as mean \pm S.E.M. values.

test in the case of an overall $P < 0.05$ in the ANOVA. Two-sided P values of 0.05 were considered significant. Individual hormone values were subjected to single cosinor analysis for characterization of diurnal rhythmicity. The results were then used to calculate the mean rhythm of inhibin B and of testosterone. Details are available in Lerchl & Partsch (23). All analyses except cosinor analysis were performed using the statistical software GRAPHPADPRISM version 2.01 for Windows (GraphPad Software Inc., San Diego, CA, USA). In general, the results are given as means \pm S.E.M. values.

Results

Hormones

After injection of 3000 IU rhFSH (Fig. 1a), serum FSH levels were significantly increased relative to baseline

levels from 4 to 96 h after injection, and maximal FSH levels (61.2 ± 5.9 U/l) could be detected after 18 h. Inhibin B levels were significantly elevated relative to baseline levels, in the morning, 24, 96 and 120 h after injection of rhFSH (Fig. 1b). Maximal levels (38% increase) were achieved after 96 h (303 ± 18 pg/ml). In addition, it was evident that inhibin followed a diurnal rhythm ($P < 0.001$) comprising higher levels in the morning and lower levels in the evening (Fig. 2a). After injection of 3000 IU rhFSH, no significant changes in serum LH and prolactin levels could be detected (data not shown). Compared with baseline values, testosterone values did not change significantly, except for a significant reduction on the evening of the second day (36 h after injection). Cosinor analysis revealed highly significant ($P < 0.001$) diurnal rhythmicity for testosterone and inhibin B (Fig. 2b). The acrophase (calculated maximum values) of testosterone at 07:38 was followed by that of inhibin B at 08:02. Likewise, the calculated

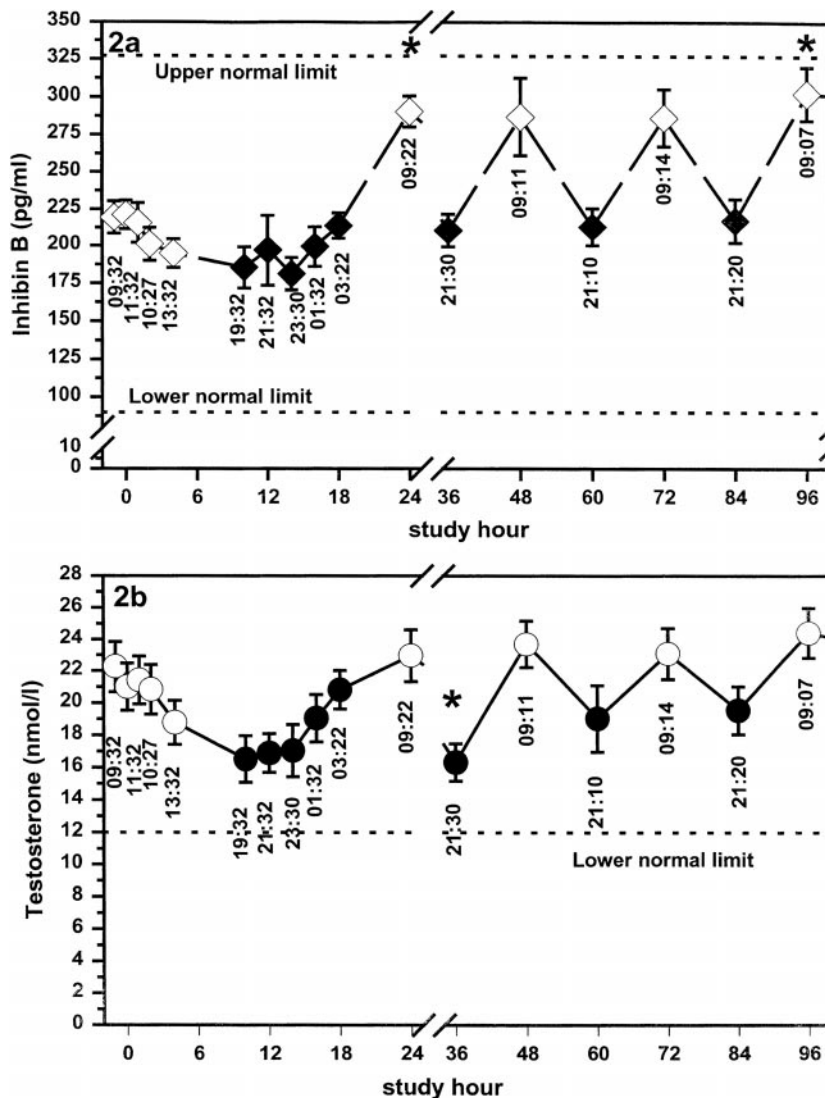


Figure 2 Serum inhibin B (a) and serum testosterone (b) levels after injection of 3000 IU rhFSH. Open symbols represent morning/daytime values and solid symbols represent evening/night-time values. The mean time of venipuncture is given on a 24 h basis. Significant differences relative to baseline values are indicated by asterisks. Results are presented as mean \pm S.E.M. values.

amplitudes of both rhythms were comparable (testosterone, 32.7%; inhibin B, 31.3%).

After placebo injection, FSH serum levels remained unchanged. After injection of 1000 and 2000 IU rhFSH, a dose-dependent increase in serum FSH levels could be detected which was significant for the 1000 IU dose from 24 to 96 h and for the 2000 IU dose from 24 to 120 h (Fig. 3). At all investigated time points, FSH serum levels were significantly different between all rhFSH doses, except for the similar FSH serum levels 120 h after injection of the 1000 IU dose and the 2000 IU dose.

After placebo injection, inhibin B serum levels remained unchanged relative to baseline levels (Fig. 4). After injection of 1000 IU and 2000 IU rhFSH, significant increases in inhibin B levels could be detected at 96 h and from 24 to 120 h respectively. Despite the significant increases seen with all rhFSH doses over time, no significant differences could be

detected in inhibin B levels between the different rhFSH doses and the placebo in the overall ANOVA, nor at any single time point investigated. However, after subtraction of baseline values, the area under the curve (AUC) after injection of 2000 and 3000 IU rhFSH was significantly higher compared with that for the placebo or 1000 IU rhFSH (Fig. 5).

At 96 h after injection of 1000 and 3000 IU rhFSH, a significant increase in inhibin pro alpha C subunits was detected relative to the baseline. Between the different rhFSH doses, and in comparison to the placebo, no significant differences in inhibin pro alpha C serum concentration could be detected 96 h after injection (Fig. 6).

Testes

Sonography of scrotal content revealed that there were no changes in testicular volume, density or structure

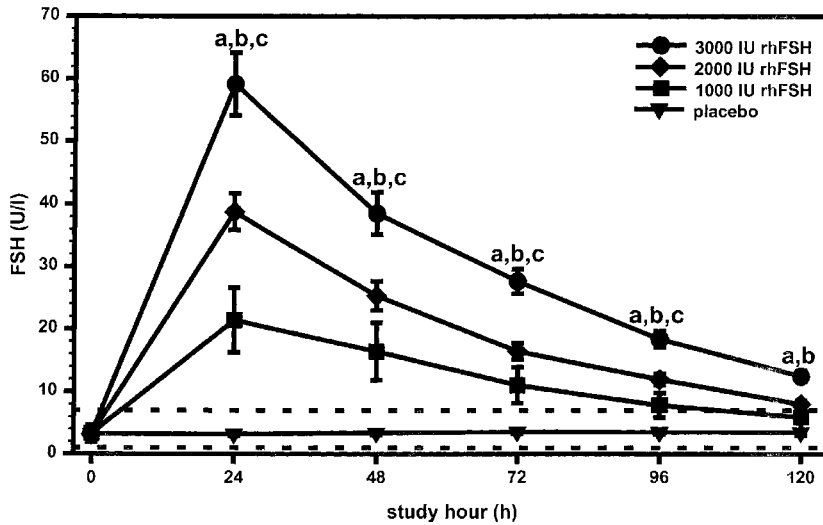


Figure 3 Serum FSH levels after injection of the placebo, 1000, 2000 and 3000 IU rhFSH. Letters represent significant differences relative to baseline values for the 3000 IU (a), 2000 IU (b) and 1000 IU (c) rhFSH doses.

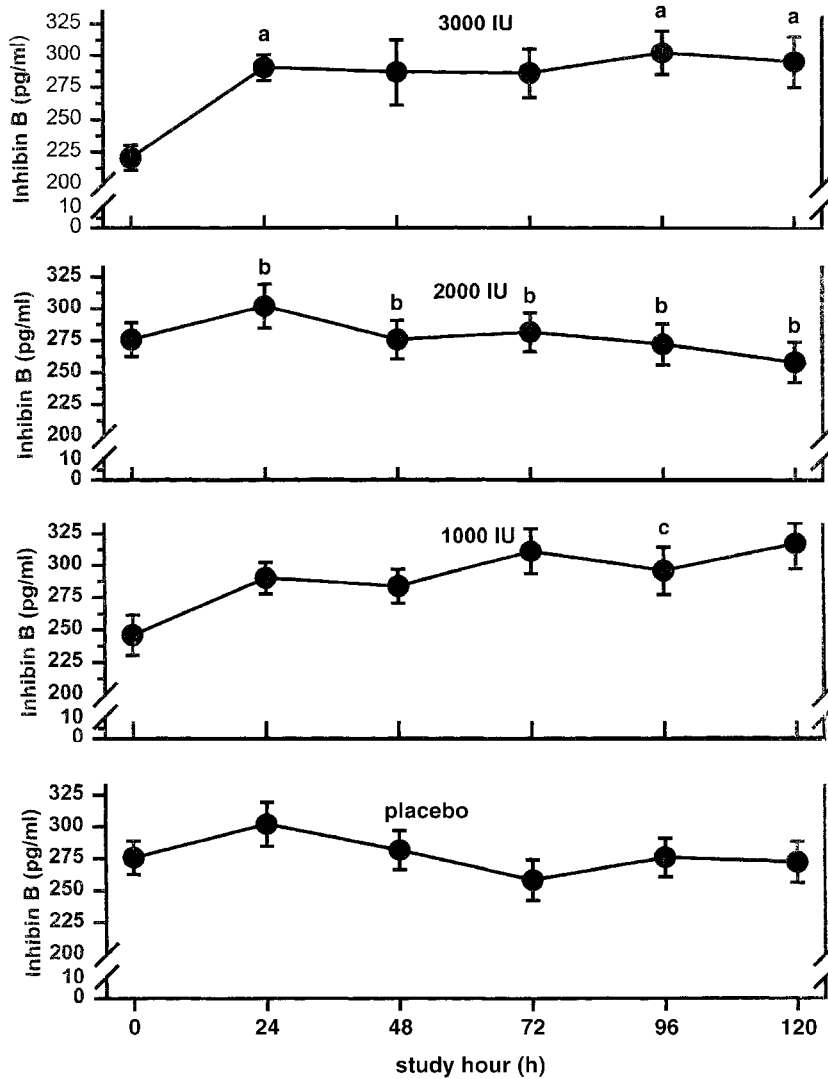


Figure 4 Serum inhibin B levels after injection of the placebo, 1000, 2000 and 3000 IU rhFSH. Letters represent significant differences relative to baseline values for the 3000 IU (a), 2000 IU (b) and 1000 IU (c) rhFSH doses.

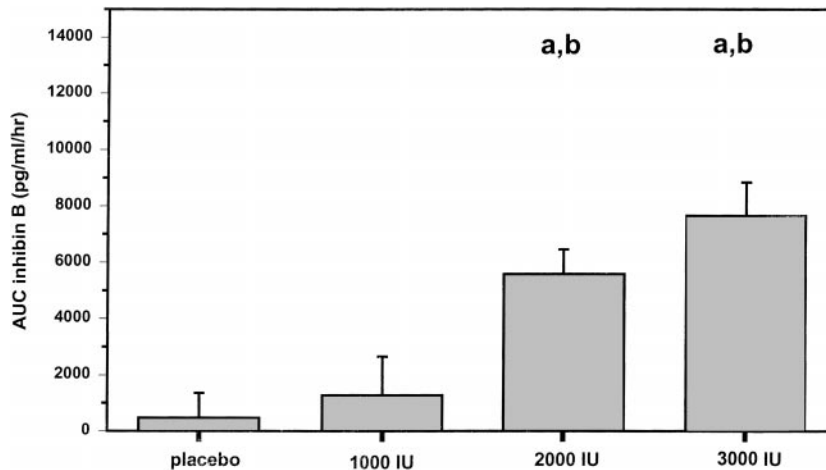


Figure 5 Area under the curve (AUC, mean \pm S.E.M.) of the serum inhibin B response after injection of the placebo, 1000, 2000 or 3000 IU rhFSH ('a' = significant compared with the placebo; 'b' = significant compared with 1000 IU rhFSH).

12, 48, 96, 144, 336 and 504 h after injection of 3000 IU rhFSH (data not shown).

Discussion

The dependence of inhibin B secretion on gonadotropin secretion was clearly demonstrated by investigating

men with hypogonadotropic hypogonadism during treatment with GnRH, thus allowing the development of the physiological inverse relationship between inhibin B and FSH levels in blood (11, 24). The gonadotropin dependence of inhibin B in the adult was further evaluated in a single-dose study in 10 normal men, using injections of 3000 IU rhFSH (3). In the

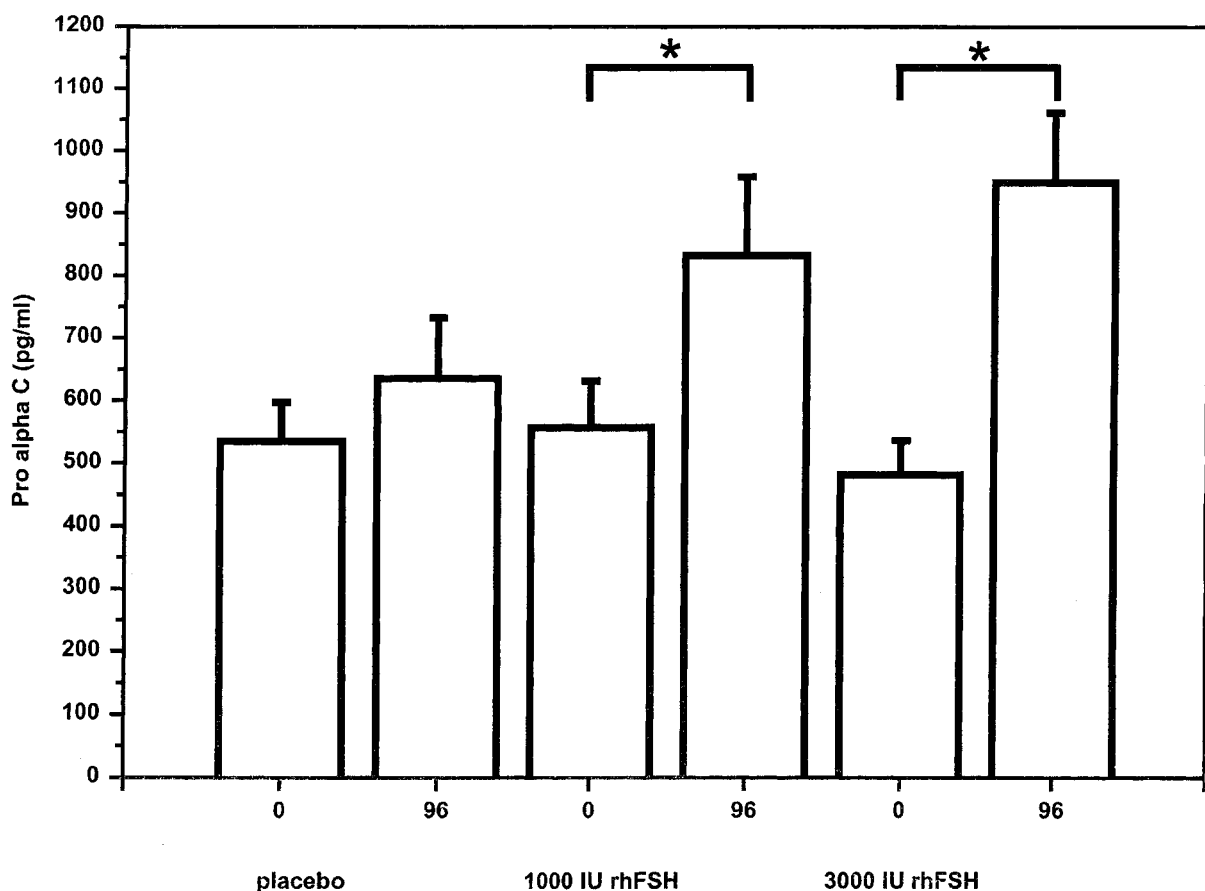


Figure 6 Serum inhibin pro alpha C after injection of the placebo, 1000 and 3000 IU rhFSH. Asterisks indicate significant differences relative to baseline values.

Anawalt *et al.* (3) study, the injection of 3000 IU rhFSH led to serum FSH levels that were much lower (peak levels 21.9 ± 3.2) than those achieved in the present study (peak levels 61.2 ± 5.9 U/l). However, the higher serum FSH levels in the present study did not result in higher inhibin B levels. The significantly increased inhibin B levels from 24 h to 7 days after 3000 IU rhFSH injection described by Anawalt *et al.* (3) were confirmed in the present study, which produced significant increases in inhibin B levels 24, 96 and 120 h after injection (Fig. 1*b*). The inhibin B peak levels achieved in both studies were very similar, though slightly higher and earlier inhibin B peaks (maximal levels of around 320 pg/ml achieved 72 h after rhFSH injection) were found in the study by Anawalt *et al.* (3) relative to the inhibin B peak levels (maximal levels 303 ± 18 pg/ml achieved 96 h after rhFSH injection) described here.

In the present study, injection of 1000 and 2000 IU of rhFSH led to a significant increase in inhibin B levels as well. Inhibin B levels increased up to a maximum of 114, 131 and 134%, relative to baseline levels, after injection of 1000, 2000 and 3000 IU rhFSH respectively. At all rhFSH doses, it is apparent that the FSH-dependent rise of inhibin B is still significant after 96 h, while FSH levels were already returning to the normal range, indicating a modest but sustained increase in inhibin B production after FSH stimulation. However, despite a clear dose-dependent increase in serum FSH levels (Fig. 3), no significant differences in serum inhibin B levels between subjects receiving the different rhFSH injections and those receiving placebo injections could be detected at any time point; after subtraction of baseline values, the inhibin B AUCs were significantly higher after injection of 3000 and 2000 IU rhFSH when compared with placebo AUCs (Fig. 5). In addition, significant differences in the AUCs for inhibin B were seen between subjects injected with 3000 and 2000 IU rhFSH and those injected with the 1000 IU dose, suggesting that inhibin B could be considered a weak pharmacodynamic parameter for FSH *in vivo*. From this study and the study by Anawalt *et al.* (3), therefore, we conclude that after exogenous injection of rhFSH a significant increase in inhibin B can be expected within 24 h of stimulation. However, the increase is modest (15–34%) and the inhibin B values after stimulation remain in the normal range. Previously, we found that the constant elevation of FSH levels obtained by daily subcutaneous administration of 150 IU rhFSH over 3 months to patients with idiopathic infertility did not influence inhibin B levels (15). However, the rhFSH dose given in the study was more than 6 times lower than the 1000 IU dose given in our current study (which barely raises inhibin B plasma levels). Furthermore, the recruitment of participants, the duration of rhFSH administration, and the basal FSH and inhibin B levels were completely different in the two studies, making comparisons difficult.

However, others have also seen significant changes in inhibin B plasma levels in infertile patients with normal FSH baseline values, after the administration of lower doses of FSH (75 IU/day) in uncontrolled clinical trials (16). Overall, these data suggest that inhibin B is at least partly dependent on FSH stimulation in fertile men. It remains to be determined whether the acute administration of rhFSH to infertile men will help to distinguish a subgroup of patients who could possibly benefit from FSH administration.

The lack of clear-cut dose-dependency for serum inhibin B levels is unexpected. Because baseline inhibin levels at the time of the first injection were lower than those at the time of the second treatment (though not significant), we cannot rule out the possibility that the previous FSH injections influenced the results of the later injections through possible effects on spermatogenesis which led to sustained inhibin B secretion. This might explain the only modest inhibin B increase after the third injection of 1000 IU rhFSH, which might remain under the influence of the first 3000 IU rhFSH injection despite a minimum of 61 days between both injections. However, one further explanation for the lack of dose-dependency might be the different physiological role of prepubertal and postpubertal germ and Sertoli cells in controlling inhibin B secretion. In newborns and in prepubertal boys, inhibin B secretion is primarily under the influence of gonadotropins (25) and both the α - and β - subunits are localized in interstitial and Sertoli cells (26). One might speculate that at this time point FSH is sufficient to regulate inhibin B production by the Sertoli cells. In the mature testis, the α -subunit is still localized in interstitial and Sertoli cells (27); the localization of the β -subunit remains controversial. It was recently reported that the β -subunit is localized in pachytene spermatocytes to early round spermatid stages and in Leydig cells and not in Sertoli cells (26). It is unlikely that a single injection of exogenous FSH (as in our study) will directly alter the number of pachytene spermatocytes to early spermatid stages or their β -subunit production. One could speculate that the production of the α -subunit is FSH-dependent and that the production of the β -subunit is regulated by signals coming from the germ cells. This would explain why inhibin B secretion can be only modestly increased by very large doses of FSH. In agreement with this hypothesis, the increase in inhibin pro alpha C (Fig. 5) after injection of 3000 IU rhFSH was almost double (197%) that observed after injection of 1000 IU rhFSH (149%).

One further explanation for the limited effect of exogenous FSH on inhibin B production might be the diurnal rhythm observed (Fig. 2*a*). Despite highly elevated FSH serum levels, the inhibin B rhythm was maintained and was similar to that of testosterone (Fig. 2*b*), the lowest inhibin B concentrations (<200 pg/ml) occurring in the evening/night-time hours. In the present study, inhibin B levels in the

late-evening venipunctures (2157 h \pm 19 min) were always lower than the morning levels (0925 h \pm 6 min) and paralleled the testosterone rhythm. The fluctuation between the low inhibin B levels in the evening and the high concentrations in the morning was higher than the increase in inhibin B above baseline levels that was caused by FSH stimulation in the morning. In addition, none of the evening inhibin B levels differed from baseline levels. Obviously, FSH injection resets inhibin B production at a higher level for several days, and the physiological diurnal rhythm of inhibin B (parallel to that of testosterone) is not influenced. This suggests that the inhibin B decrease in the evening is not dependent on FSH, which, in this case, remains constantly high. This also explains why, after injection of 3000 IU rhFSH, initially, during the day, a decrease in inhibin was observed, which is in accordance with the inhibin B diurnal rhythm.

The diurnal rhythm of inhibin has been described previously by other groups (28–31). Our study confirms the data of Carlsen *et al.* (31), who found no correlation between serum FSH and the diurnal rhythm of inhibin B, suggesting that inhibin B might be regulated by other gonadal factors. Although Leydig cells express α - and β B-subunits, they are not the source of inhibin B in adults, since recombinant, human LH administration does not increase inhibin B serum levels (32). Carlsen *et al.* (31) showed that testosterone levels had a significant influence on the level of inhibin B, with a time lag of 0.5–1 h, whereas inhibin B did not influence the levels of testosterone. Our study confirms the results of Carlsen *et al.* (31), as the mean time lag between the calculated acrophases for testosterone and inhibin B in our study was 24 min. It might well be that an unknown mechanism underlies both rhythms, which are not influenced by FSH or LH. In addition, it has been suggested that FSH modulates the production of testosterone by the Leydig cells through a non-steroidal factor released by the Sertoli cells (33, 34). However, in view of the intensive paracrine interaction between Leydig cells and Sertoli cells, and, as the androgen receptor is expressed in Sertoli cells, it may well be that testosterone has a modulating effect on the stimulation of inhibin B production in the Sertoli cell.

In conclusion, our results and the findings of others, summarized in a recent review (1), support the hypothesis that inhibin B is an index of global testicular function whose secretion reflects a fundamental interaction between germ cells, FSH and Sertoli cells and, possibly, Leydig cells. In the normal adult male, FSH can stimulate inhibin B by raising the set point for its production by Sertoli cells without interfering with its diurnal rhythm. Therefore, inhibin B should be considered only as a partial pharmacodynamic parameter of FSH *in vivo*, the integrity of the spermatogenic process being the second fundamental component in the regulation of its secretion in blood.

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