Inhibin, a dimeric glycoprotein hormone, is thought to play an important role in the biological feedback system of FSH release from the pituitary gland (1, 2). In the male, in vitro studies have shown that the Sertoli cell is the primary source of testicular inhibin (3, 4). According to De Kretser et al. (5) and Drummond et al. (6), the Leydig cell should not play a relevant role for inhibin concentrations in the peripheral blood. In men, there is also evidence that immunoreactive inhibin (ir-inhibin) is co-secreted with testosterone into the spermatic venous blood in a pulsatile manner (7). In the peripheral circulation, a pulsatile secretion pattern of ir-inhibin has not been detected until now. In a number of species inhibin was isolated and its chemical structure identified (8–11). Australian and Japanese groups succeeded in isolating and purifying bovine inhibin from follicular fluid and established a RIA for measuring inhibin concentrations in biological fluids (9, 12). These widely used RIAs were based upon cross-reactivity of antiovine inhibin antibodies with human inhibin. A serious limitation of these assays was a relatively low specific binding of the antibody to human inhibin. The development of a sensitive two-site immunoenzymatic assay (13) with antibodies raised against distinct epitopes of the α-chain of human inhibin allows us to measure ir-inhibin in secretion profiles of healthy male volunteers over a period of 24 h. In this investigation, we attempted to clarify the secretion pattern of ir-inhibin in the peripheral blood from healthy male volunteers using the aforementioned sensitive assay system for human ir-inhibin and its dependence on the secretion of testosterone and the gonadotropins.

Subjects and methods

Subjects and samples

Five healthy male volunteers, aged 24–27 years (medical students in our university), were investigated...
to observe chronological changes in serum concentrations of ir-inhibin with regard to testosterone, LH and FSH levels, respectively. They all passed physical and especially andrological examinations, including biochemical studies. No pathological results were found. A catheter was inserted into the cubital vein at 08.00 h, and blood samples were collected every 15 min over a period of 24 h. Blood was centrifuged at 3000 g for 15 min and the serum was separated and stored at -20°C until assay. During this investigation, no restrictions regarding meals and sleep were made. The study was approved by the ethical committees of the University of Bonn, Germany.

**Hormone analyses**

Immunoreactive inhibin has been determined by a two-site immunoenzymatic assay (Medgenix Diagnostics, Fleurs, Belgium), as described previously (14). The assay utilizes two antibodies that are directed against the 1–32 epitopes on the α-subunit of human inhibin: a goat polyclonal antibody coated on the wells of the microtiter plate (amino acids 15–32) and a mouse monoclonal antibody conjugated to peroxidase (amino acids 1–17). The inhibin standard is a preparation of pooled human follicular fluid obtained from patients undergoing hyperstimulation in an in vitro fertilization program, because no purified human inhibin from a natural source has been available. This internal standard for inhibin has been calibrated against an arbitrary unit system: 1 U/1 x 10⁻³ is defined as the median value of circulating serum inhibin in 30 healthy male subjects; 1 unit is equivalent to 400 pg of recombinant α-βA human inhibin 32 kd as well as to 143 units of WHO/NIH porcine inhibin standard MRC 86/690 (Professor Franchimont, Liege, Belgium; pers. comm.). Normal men had ir-inhibin concentrations of 1.77 ± 0.09 U/l x 10⁻³ (range 0.79–3.1, N = 40), as described previously (14). All samples were assayed in duplicate; the sensitivity of the assay was 0.1 U/l x 10⁻³. The within- and between-assay cvs were 6.5% (N = 20) and 8.3% (N = 12), respectively. Serum from a normal healthy man produced dose–response lines that paralleled that of the standard curve. No specific binding of the antiserum was observed in the serum from a postmenopausal woman (Fig. 1). The free α-subunit of inhibin also was detected in this assay. No significant interferences were described with the other inhibin-related proteins, like activin, transforming growth factor beta or LH, FSH, follistatin and seminal inhibin-like peptide.

The LH, FSH and testosterone were determined by commercially available IRMA (LH, FSH; Serono, Freiburg, Germany) and RIA (testosterone; RSL, ICN Biomedicals Inc., Costa Mesa, CA, USA). The normal range in men for LH is 1.8–9.2 IU/l, for FSH is 1.6–9.7 IU/l and for testosterone is 10.4–34.7 nmol/l. The within-assay variation for LH, FSH and testosterone was 3.5%, 3.9% and 9.5%; the between-assay variation was 9.4%, 5.3% and 9.8%, respectively.

**Statistical analyses**

Hormone concentrations were presented as median

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<td>1.0 Inhibin</td>
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<td>2.4</td>
<td>1.8</td>
<td>2.6</td>
<td>2.0</td>
</tr>
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<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Theta (h)</td>
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<td>12.2</td>
<td>15.0</td>
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<td>13.4</td>
</tr>
<tr>
<td>F statistic</td>
<td>51.2*</td>
<td>10.6*</td>
<td>119*</td>
<td>45.7*</td>
<td>28.5*</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Case no.</th>
<th>1</th>
<th>2</th>
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<td>15.8</td>
<td>19.1</td>
<td>19.9</td>
</tr>
<tr>
<td>Amplitude</td>
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<td>5.7</td>
<td>3.2</td>
<td>0.9</td>
<td>4.1</td>
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<tr>
<td>Theta (h)</td>
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<td>9.1</td>
<td>9.0</td>
<td>9.1</td>
<td>8.6</td>
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<tr>
<td>F statistic</td>
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<td>55.4*</td>
<td>29.6*</td>
<td>2.6 (NS)</td>
<td>29.1*</td>
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*p < 0.05.
Fig. 2. Time courses of serum LH (●) and FSH (○) concentrations (upper panel) and of serum immunoreactive inhibin (ir-inhibin) (●) and testosterone (○) concentrations (lower panel) in five healthy men, expressed as median values. 

Statistical analyses were performed using Pearson’s correlation coefficient (r). A p value lower than 0.05 was considered significant. Moment to moment changes in ir-inhibin were performed with the Cluster analysis computer algorithm provided by Veldhuis (17), which was set to detect nadir and peak clusters of two samples, each with a t statistic for up and down strokes of 2.0. The 24-h concentration profiles of testosterone and ir-inhibin were tested for diurnal variation by cosinor rhythmometry, a computer program provided by Dr W Burr (Clinic of Epileptology, University of Bonn) as described previously (18). A significant fit of the curve was defined when the possibility of the data representing a horizontal line rather than a cosine curve was less than 5%. The acrophases, which were not necessarily the detected maximal levels, represent the time of occurrence of the maximal values of the best-fitting pattern; the amplitude was defined...
Fig. 3. Time courses of individual serum immunoreactive inhibin (Ir-inhibin) (●) and testosterone (○) in five healthy volunteers. Moment to moment changes of Ir-inhibin scored as pulses by Cluster analysis are marked by asterisks (+).
as 50% of the difference between the acrophase and nadir levels.

Results

Median serum LH and FSH concentrations over 24 h in five healthy male volunteers are illustrated in Fig. 2 (upper panel). Gonadotropin concentrations detected were in the normal range (LH: range 0.9–6.1 U/l; FSH: range 2.5–7.2 U/l) and varied depending on the time and the person under observation. No diurnal variation of gonadotropins was observed.

Immunoreactive inhibin concentrations were within the normal range (0.79–3.1 U/l × 10⁻³), as described previously (14). The highest ir-inhibin concentrations were observed at 08.00 h in the morning, with peak values of 2.45–3.20 U/l × 10⁻³; the lowest values were measured between 01.00 h and 02.00 h (1.20–1.86 U/l × 10⁻³). The ir-inhibin concentrations remained relatively constant during the day, followed by a gradual decline at night and coming to a nadir between 01.00 h and 02.00 h. After the nadir, ir-inhibin increased gradually to the initial level between 06.00 h and 08.00 h in the morning.

Testosterone concentrations varied between 7.35 and 36.6 nmol/l; at night, testosterone concentrations were in the lower normal range or subnormal (7.35–12.6 nmol/l), with a significant increase in the early morning at 06.00 h (20.5–36.6 nmol/l, p < 0.01).

Secretion profiles of ir-inhibin and testosterone concentrations of the volunteers investigated—expressed as medians—are shown in Fig. 2 (lower panel). A significant correlation was found between median ir-inhibin and testosterone concentrations (r = 0.449, p < 0.001). The correlation between serum ir-inhibin and testosterone analyzed individually is shown in Table 1. A slight significant correlation was found between individual ir-inhibin
and testosterone concentrations in four of the five subjects. There was no correlation between ir-h inhibin, LH and FSH; in particular, no inverse relationship between ir-h inhibin and FSH could be observed in adult healthy men.

Individual ir-h inhibin and testosterone concentration profiles over a period of 24 h of the five volunteers are shown in Fig. 3. A significant diurnal secretion of ir-h inhibin in the five volunteers was validated by cosinor-rhythmometry, as well as a circadian testosterone secretion mode in four of the five volunteers (Table 2).

Moment to moment changes scored as pulses by the Cluster algorithm (16) were marked by asterisks (Fig. 3). The Cluster pulse analysis computer algorithm identified four to seven pulses of ir-inhibin per day, depending on the person under observation, as shown in Fig. 3. No coincidences of ir-inhibin fluctuations in the five subjects could be observed, but an increase of the frequency of fluctuations in ir-inhibin concentrations seemed to be imposing in the period of decrease during the evening in four of the five subjects.

Discussion

The present investigation demonstrates a clear circadian rhythm of ir-h inhibin and testosterone in healthy men, validated by cosinor rhythmometry. These results are in line with Yamaguchi and co-workers (19), who described a circadian secretion of inhibin in normal men. A remarkable finding in the present investigation was that an ultradian secretion pattern of ir-inhibin exists in the peripheral circulation overlaying the diurnal discharge. These data are in line with Winters (7), who found by serial inhibin measurements that inhibin release was pulsatile in spermatic vein blood, but obviously not in the peripheral blood. Cluster analysis in this investigation identified 2.8 ± 0.5 pulses/4 h. In our trial, Cluster analysis identified 4–7 pulses/24 h. The reason for this discrepancy may be an effect of dilution in the peripheral blood; the peripheral inhibin concentrations are four times lower than in the spermatic veins (7). In line with Winters (7), our results indicate that ir-inhibin is co-secreted particularly with testosterone. Nevertheless, for the first time we were able to identify moment to moment changes of ir-inhibin in the peripheral blood in healthy men. Ultradian changes of ir-inhibin concentrations have not been observed by Yamaguchi and co-workers (19), but, in contrast to our investigation, blood was taken every hour over a period of 24 h. The different results of our investigation regarding the interval of detecting ir-inhibin (1 h vs 15 min) could be explained by the possibility that moment to moment changes of ir-inhibin remained undetected in this former trial.

In line with several authors (20–24), a diurnal pattern of testosterone secretion was quite evident, which explained the wide variation of testosterone concentrations measured: although a circadian rhythm in testosterone secretion did not occur in all healthy men. We analyzed a positive relationship between ir-inhibin and testosterone concentrations in four of five healthy men. These results are in line with Yamaguchi et al. (19), who found a significant correlation between these testicular hormones in three of five normal men.

The Leydig cells might be involved in the regulation of inhibin synthesis and/or secretion from the Sertoli cells but testosterone did not play a direct stimulatory role in the inhibin release, as described previously (6, 26). It is evident also that small amounts of immunoreactive inhibin, inhibin α-subunit proteins and mRNAs for the inhibin subunits were detected in Leydig cells of the rat testis and in Leydig cell-derived cell lines (27). According to several authors (5, 6), the Leydig cells are not playing a relevant role with respect to inhibin concentrations in the peripheral blood.

No inverse relationship was found between the secretion pattern of ir-inhibin and FSH in healthy men, as described previously (19). This discrepancy to the proposed inhibin physiology might be due to the long half-life of FSH (25) and the involvement of other hypothalamic factors in the release of FSH.

The enzyme immunoassay used may also detect the free α-subunit of inhibin and its precursor proteins in the peripheral blood. Recently, Robertson et al. (15) described the isolation of an α-subunit-derived dimeric protein (termed pro-α) from bovine follicular fluid, which significantly cross-reacts in the widely used inhibin RIA. Schneyer et al. (16) showed the presence of inhibin α-subunit in human serum by Western blot, suggesting that data detected by RIA should be interpreted with care. This may be a serious limitation in the immunoenzyme assay, too. The development of specific immunoassays detecting dimeric human inhibin is required, which would help to understand the complex regulation of inhibin release. The etiology of the circadian ir-inhibin and testosterone serum changes remains unknown. Physiological factors, such as changes in hemodynamics simulating a diurnal and pulsatile secretion pattern of ir-inhibin, were not measured in the present investigation and could not be excluded.

In conclusion, we demonstrated that the secretion pattern of ir-inhibin in the peripheral circulation of healthy men followed a diurnal rhythm correlation to testosterone secretion; this circadian mode of ir-inhibin secretion is accompanied by moment to moment changes. The physiological role of this ultra- and circadian release of inhibin was not clarified in this study and requires further investigations.

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References

Inhibin—fact or artifact. Mol Cell Endocrinol 1979;1:31–10

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