Immunoneutralization of follicle stimulating hormone does not affect gonadotrophin surge-inhibiting factor/attenuating factor bioactivity during the rat ovarian cycle

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The physiological role of follicle stimulating hormone (FSH) in the regulation of the release of the putative ovarian factor gonadotrophin surge-inhibiting or -attenuating factor (GnSIF/AF) was investigated. Blood FSH concentrations were immunoneutralized in female rats during different days of the ovarian cycle. FSH antisera was injected in different protocols to neutralize the FSH surge(s) and/or basal FSH concentrations. All animals were killed on the morning of the day of pro-oestrus of the control rats. Hemi-pituitaries were incubated and gonadotrophin-releasing hormone (GnRH) stimulated and basal luteinizing hormone (LH) and FSH releases and pituitary contents were measured. The biological effectiveness of the FSH antiserum was established by monitoring the histology of vaginal smears and inhibin concentrations in trunk-blood. The predominance of small leukocytes in the smears (low oestradiol bioactivity) and the decreased (bioactive) inhibin secretion imply that anti-FSH neutralizes FSH bioactivity. Simultaneously, the results showed that all anti-FSH injections did not affect the biphasic LH response to GnRH in vitro, which shows an unaltered presence of GnSIF/AF bioactivity in the blood circulation. The results suggest that under physiological conditions GnSIF/AF bioactivity, which keeps the LH responsiveness of the pituitary gland suppressed to the action of GnRH, is not supported by inhibin and not controlled by FSH.

Key words: functional antagonism/GnRH/hyperstimulation/ luteinizing hormone/self-priming

Introduction

A well-timed sequence for the resumption of meiosis and the induction of ovulation during the LH surge optimizes oocyte viability and improves the chances of successful conception and pregnancy outcome (Regan et al., 1990; Mattheij et al., 1994; see also de Koning, 1995). The timely excesses of low and high luteinizing hormone (LH) threshold concentrations in blood are responsible for the initiation of these processes. To prevent deterioration of the oocyte, LH concentration before the onset of the LH surge should be kept below the threshold for the resumption of meiosis. This is achieved by a functional antagonistic system involving the putative ovarian factor gonadotrophin surge-inhibiting or -attenuating factor (GnSIF/AF; Littman and Hodgen, 1984; Messinis and Templeton, 1990, 1991; de Koning, 1995; Fowler and Templeton, 1996) and gonadotrophin-releasing hormone (GnRH).

To increase the rate of LH release via the so-called GnRH self-priming process, GnRH-induced de-novo protein synthesis plays a mandatory role (de Koning et al., 1976; Pickering and Fink, 1976). The synthesized protein (also named self-priming-associated protein or SPAP) is the rate-limiting step in LH release. Initially SPAP bioactivity is low, but after GnRH stimulation its synthesis is increased and allows an increase in the rate of GnRH-stimulated LH release. Therefore, during repetitive or continuous GnRH stimulation this results in the well-known biphasic LH release pattern (for review see de Koning, 1995). GnSIF/AF prevents or eliminates GnRH-induced increased SPAP bioactivity during the major part of the cycle by keeping the pituitary LH responsiveness to GnRH low (de Koning et al., 1987a,b, 1989, 1991; Busbridge et al., 1990; Whitehead, 1990; Knight et al., 1990; Koppenaal et al., 1991, 1992; de Koning, 1995; Fowler and Templeton, 1996). This is the underlying cause of the initial low rate of LH release in response to GnRH. If the influence of GnSIF/AF wears off, as happens after ovariectomy (OVX), SPAP bioactivity increases. Thus, a moderate increase in SPAP elevates the initial phase of the biphasic LH response to GnRH, but a further increase, as after long-term OVX, allows an immediate high monophasic LH response (Koppenaal et al., 1992).

Administration of follicle stimulating hormone (FSH) suppresses GnRH-stimulated LH release in women and in female monkeys and rats (Geiger et al., 1980; Littman and Hodgen, 1984; Messinis and Templeton, 1990, 1991; Koppenaal et al., 1991, 1992; de Koning et al., 1994; de Koning, 1995; Fowler and Templeton, 1996). It was hypothesized that this has to be caused by the increased production of GnSIF/AF. In one of these experiments FSH-induced increased GnSIF/AF bioactivity was measured by estimating the SPAP bioactivity (Koppenaal et al., 1992). For this, a qualitative bioassay was applied in which these pituitaries were exposed to a high concentration of GnRH in vitro. This method allows the detection of small changes in the bioactivity of SPAP that are inversely related to GnSIF/AF bioactivity in vivo (de Koning et al., 1977, 1979, 1980; Koppenaal et al., 1992).

To confirm a regulatory role for FSH on LH release during the rat ovarian cycle, we studied the effects of in-vivo immunoneutralization of circulating FSH on the pituitary LH responsiveness to GnRH using the above in-vitro design. It is expected that such a design will cause the opposite effect to
those from the administration of FSH. Also measured were the biological effects of changes in oestriadiol (vaginal smears) and inhibin (in-vitro release of FSH) which are also controlled by FSH (McLachlan et al., 1988; Hee et al., 1993; Muttukrishna et al., 1994). If GnSIF/AF bioactivity during the cycle is controlled by FSH, GnSIF/AF concentrations will fall and the SPAP bioactivity will increase. During incubation with GnRH, this will result either in a biphasic LH secretion pattern in which the initial phase is increased, or in an increased monophasic, OVX-like, pattern.

Materials and methods

Animals

Female rats from the Wistur-derived colony kept in this laboratory were allowed free access to food and water in an animal room illuminated from 07.00 to 19.00 h and were kept at a constant temperature of 22°C. Vaginal smears were taken daily and only regular 4 day cyclic rats (180–200 g) were used. After anti-FSH injections vaginal smears showed dioestrous patterns (e.g. predominance of small leukocytes) which is indicative of low oestriadiol blood concentrations.

Immunoneutralization of FSH

Rabbit anti-ovine FSH serum was used to neutralize endogenous FSH bioactivity. In a previous study, injections of this antiserum in female rats have been proven to block the action of endogenous FSH (Welschen and Dullaart, 1976; see below). The vaginal histology (absence of oestriadiol bioactivity) agrees with these observations. Four different protocols of antibody administration were applied (see also Figure 1).

Protocol 1: one injection (0.1 ml) was given on the day of pro-oestrus of the preceding cycle (assumed neutralization of the FSH surges). Protocol 2: one injection (0.1 ml) was given on day 1 of dioestrus (assumed neutralization of basal FSH concentrations). Protocol 3: two injections (0.2 ml each) were given successively on the days of pro-oestrus (of the preceding cycle) and of oestrus (assumed neutralization of the FSH surges). Protocol 4: four injections (0.2 ml each) were given successively on the days of pro-oestrus (of the preceding cycle), oestrus, and on days 1 and 2 of dioestrus (assumed neutralization of FSH during the whole cycle).

All rats were injected i.v. at 10.00 h. Because of the absence of any effect on LH release in vitro, the amount and number of the antiserum injections were increased (see below). Control rats received the same injections but with normal rabbit serum (NRS). The experiments, and also part of the experimental groups, were carried out separately. However, a treated group was always compared with matched controls in one experiment.

Pituitary incubations

All animals were killed by decapitation in the morning of the day of pro-oestrus of the control rats between 10.00 and 10.30 h, which was 1–4 days after the (last) injection of anti-FSH (see protocols inserted in Figure 1). Each anterior pituitary gland was halved and both halves were placed into a single well of a 24 multi-well dish (Costar, Cambridge, MA, USA) containing 1 ml ice-cold medium TC 199 (Boehringer, Mannheim, Germany) and preincubated for 30 min. The incubations were carried out at 37°C with continuous shaking in an atmosphere of 95% O2:5% CO2. After preincubation, the culture medium was replaced by fresh medium with or without 1 μmol/l GnRH (Boehringer). To avoid interference of the results by possible changes in the sensitivity of the fresh pituitary glands to GnRH or by degradation of GnRH, the concentration of GnRH was about \( 100 \times \text{dose needed to achieve a maximal LH release (de Koning et al., 1976).} \) Thus, the initial part of the GnRH-induced LH secretion pattern reflects the maximal responsiveness of the pituitary gland to GnRH (de Koning et al., 1976, 1977, 1979, 1980).

Samples of 100 μl were withdrawn from the medium after 1, 2 and 4 h of incubation. After termination of the incubation, LH and FSH were extracted in 0.9% (w/v) NaCl by homogenization of pituitary tissue with a glass–glass potter apparatus. If the pituitary glands were incubated in medium only, the incubation lasted for 8 h and the samples were withdrawn after 1, 2, 4 and 8 h. Media and extracts were assayed for LH and FSH.

Hormone analysis

The LH and FSH estimations were carried out according to de Koning et al. (1987a). Rat LH- and FSH-I–6 and LH-RP-3 and FSH-RP-2 (kindly provided by the NHP, NIDDK and NICHD, USA) were used for iodination and as standard preparations, respectively. The sensitivity of the assays, defined as the standard concentration required to suppress binding of iodinated LH or FSH to 90% of that occurring without unlabelled LH or FSH was estimated as 72 ± 18 (SD) pg/tube \((n = 20)\) or 42 ± 4 pg/tube \((n = 20)\) respectively. The intra- and interassay coefficients of variation for LH were 8.1 and 11.4% respectively, for FSH 6.2 and 16% respectively. LH and FSH concentrations were corrected for volume loss due to sampling during the experiment.

Immunoreactive inhibin in plasma was measured by radioimmunoassay as described by Van Cappellen et al. (1995), using an antisem against purified 32 kDa bovine follicular fluid inhibin and iodinated 32 kDa bovine follicular fluid inhibin. All samples were measured in one assay. The intra-assay coefficient of variation amounted to 17.5%. A charcoal-treated bovine follicular fluid preparation with an arbitrary potency of 1 U/mg protein was used as a standard for inhibin.

To avoid interference of the rabbit anti-ovine FSH serum or NRS in the assays, 150 μl samples of rat trunk blood were pretreated with excess (250 μl) of a 10% heat-inactivated Staphylococcus aureus suspension. The antibody–S. aureus complex was removed by centrifu-
FSH may not regulate GnSIF/AF release

Figure 2. Effects of anti-follicle stimulating hormone (FSH) injection(s) on the subsequent response of pituitary glands during incubations in the presence (a, b) or absence (c) of gonadotrophin-releasing hormone (GnRH). Cumulative release of luteinizing hormone (LH) (a) and FSH (b) by hemi-pituitary glands during a 4 h incubation period with 1 µmol/l GnRH (mean ± SEM; n = 4–5). The glands were collected at 10.00 h on the day of pro-oestrus (p) from adult rats that had received 0.1 ml anti-FSH (all interrupted lines) on p (△), 0.1 ml anti-FSH on dioestrus 1 (d1; □) or 0.2 ml anti-FSH on p, o, d1 and dioestrus 2 (d2; ○). Appropriate control rats received normal rabbit serum (– – – –; n = 16). (c) Cumulative basal release of LH (●) and FSH (●) in vitro (mean ± SEM; n = 6) by pituitary glands taken at 10.00 h on the day of pro-oestrus from adult rats that received 0.2 ml anti-FSH on p, o, d1 and d2 (– – – –). Control rats received NRS (●).

Regulation. *S. aureus* contains protein A in its cell membrane that has a high affinity for these immunoglobulins.

Statistical analysis

Statistical comparisons were made by analysis of variance (ANOVA) and followed by Duncan’s multiple comparison test (Steel and Torrie, 1960). If the data showed heterogeneity of variance, logarithmic transformation was carried out before statistical analysis. A difference was considered significant when ANOVA showed a significant heterogeneity among all groups and the multiple comparison test gave a value of $P < 0.05$ for the groups concerned.

Results

**FSH plasma concentrations during a normal ovarian cycle**

Figure 1 shows the FSH plasma concentrations during a normal ovarian cycle of the rat: high from the afternoon of pro-oestrus until the day of oestrus, and low during the other days. Figure 1 also includes the four protocols for the injections of anti-FSH (~10.00 h) for the immunoneutralization of the blood concentrations of FSH (see Materials and methods).

**Pituitary LH and FSH responses to GnRH in vitro**

Adult female rats were treated with anti-FSH serum or NRS according to the four protocols. After anti-FSH injections vaginal smears showed dioestrous patterns (predominance of small leukocytes) which is indicative of low oestradiol blood concentrations. The pituitary glands were collected and incubated in the presence of 1 µmol/l GnRH to assay SPAP bioactivity, the rate-limiting step in LH release (see de Koning et al., 1977, 1979, 1980).

Figure 2a shows the cumulative GnRH-induced release of LH. The slopes of the secretion patterns reflect the secretion rates of LH. In all cases, biphasic LH secretion patterns were displayed: an initially low rate of LH release (lag-phase) was followed by an increased rate of LH release (primed-phase). Such a biphasic LH secretion pattern in response to this high concentration of GnRH, indicated a low SPAP and a high GnSIF/AF bioactivity in vivo at the time of killing of the rats. The second phase was due to the de-novo synthesis of SPAP during incubation. Since no significant differences were observed between the different NRS controls, their total mean value is given here and below. Between the data of each protocol, no statistical differences were observed between the GnRH-stimulated LH secretion patterns after the anti-FSH or the appropriate NRS treatments.

The cumulative GnRH-induced release of FSH was measured in the same media of the above experiments. In NRS-treated rats, a significant biphasic FSH secretion pattern in response to GnRH was displayed (Figure 2b). Pituitary glands from all anti-FSH serum-treated rats displayed significantly increased GnRH-stimulated FSH secretion rates. The typically biphasic release patterns largely disappeared in response to the multiple anti-FSH injection protocols.

**Unstimulated LH and FSH release in vitro**

Pituitary glands from another group of adult female rats treated with 0.2 ml anti-FSH or NRS according to the fourth injection protocol were incubated in medium without GnRH (Figure 2c). No significant differences were observed between the cumulative basal LH concentrations in the NRS- and anti-FSH-treated rats.

In comparison with the NRS controls, anti-FSH significantly increased the rate of constitutive FSH release, which thus reflects decreased inhibin bioactivity at the time of killing of the rats.
S.Tio, J.A.M.J.van Dieten and J.de Koning.

Figure 3. Effect of anti-follicle stimulating hormone (FSH) (narrow hatched bars) or normal rabbit serum (open bars) treatment in vivo on total pituitary luteinizing hormone (LH) (a) and FSH (b) contents, measured at the day of pro-oestrus after termination of the 4 h incubation with gonadotrophin-releasing hormone (GnRH). Results (mean ± SEM) are from the same experimental groups as displayed in Figure 2a and b. 0.1 ml injections were given on pro-oestrus of the preceding cycle (p) or on dioestrus 1 (d1), and 0.2 ml injections on pro-oestrus of the preceding cycle and on dioestrus (p/o), or on p/o and on the days of dioestrus 1 and 2 (p-d2). *Significantly different from all anti-FSH-treated groups (P < 0.05, Duncan’s multiple comparison test).

Total pituitary LH and FSH contents

After incubation of the pituitary glands, their LH and FSH contents were determined. The total LH content was calculated from these values to which were added the amounts of hormone released into the media during incubation. No significant differences were observed between the LH contents of the anti-FSH- and NRS-treated groups (Figure 3a). In comparison with the NRS-treated groups of all protocols, a significant increase in total pituitary FSH contents was seen in all anti-FSH treated rats (P < 0.05; Figure 3b).

Serum concentrations of LH and inhibin

Serum hormone concentrations were determined on the morning of pro-oestrus at 10.00 h. The values of LH increased significantly after anti-FSH treatment (P < 0.05). This increase was highest after the multiple anti-FSH injection protocols in which also the amount of antiserum was doubled per injection (Figure 4a).

Inhibin concentrations were significantly decreased only after the multiple anti-FSH injection protocols (P < 0.05; Figure 4b). The results from the multiple antiserum injection protocols also differed significantly from those of the single antiserum injection protocols (P < 0.05).

Discussion

As mentioned in the Introduction, injections of FSH suppress and prolong the initial phase of the well-known biphasic LH secretion pattern in response to GnRH, which was taken as an indirect measure of increased GnSIF/AF bioactivity. In contrast, an immediate increased LH response to GnRH is noted after a short- or long-term absence of the ovaries (i.e. GnSIF/AF). This varies between a moderate increased LH response and the maximal possible second phase response, respectively. These changes in the LH release patterns reflect the initial height of the pituitary SPAP concentrations and the time necessary for its synthesis, the rate-limiting step in LH release. The underlying cause of the changes in the initial SPAP concentrations is assumed to be the increased or eliminated production of GnSIF/AF after FSH administration or OVX, respectively. Thus, it will take more (after FSH) or less (after short-term OVX) time for GnRH to stimulate the de-novo synthesis of SPAP to increase the rate of LH release (Koppenaal et al., 1992; de Koning et al., 1994). After long-term OVX, SPAP concentrations are already maximal and no further SPAP synthesis is required. It may be concluded that normally during
the cycle the biological effect of GnSIF/AF is submaximal. Otherwise, FSH and OVX would have been without any effect.

The rationale behind the present experimental design is based on these observations. The applied in-vitro system detects small changes in the maximal pituitary LH response secondary to changes in the SPAP activity (de Koning et al., 1977, 1979, 1980, 1987b). Besides this, the method has also proven its validity in one of the above-mentioned FSH-stimulation studies (Koppenaal et al., 1992).

It was expected that immunoneutralization of FSH and thus the discontinuation of GnSIF/AF release would cause the opposite biological effects of FSH administration on oestradiol, inhibit and GnSIF/AF bioactivities. Hormone binding by antibodies, however, does not necessarily result in neutralization of its bioactivity. From studies in which this (Welschen and Dullaart, 1976) or another (Schwartz et al., 1973) anti-FSH preparation was used, the effectiveness in neutralizing the biological activity of circulating FSH was shown by vaginal smears and ovarian histology, oestradiol release and oestrogen secretion. In short, in the experiments of Welschen and Dullaart (1976) this anti-FSH (20–720 µl) was injected once in cyclic rats at noon on the day of pro-oestrous to suppress the high FSH surges (see Figure 1). The rats were killed 1, 3 or 5 days later. One day after the injections, a considerable decrease in number of small follicles to Graafian follicles by all dosages was observed, whereas no effect on oestradiol was found. One injection of 80 µl anti-FSH showed the formation of an abnormal pattern of follicle development during later days.

Since no effects were found of single anti-FSH injections on the pituitary LH response to GnRH, higher amounts of this antiserum and more injections until the day of the experiment were given. The effectiveness of the antibody in neutralizing the biological action of FSH is shown by (i) the predominance of small leukocytes in vaginal smears (indicative of decreased blood concentrations of oestradiol), (ii) the increased constitutive release of FSH in vitro (indicative for the decreased release of bioactive inhibit) and (iii) the suppression of inhibit plasma concentrations.

After cessation of the GnSIF/AF release, its biological impact on the pituitary LH responsiveness in rats disappears within a few hours (de Koning et al., 1979, 1980; Van Dieteren and de Koning, 1995). However, no matter which anti-FSH serum injection protocol was applied, GnRH-stimulated LH release by the pituitary glands was not affected: in all cases unaltered normal biphasic LH release patterns were displayed. This shows that neutralization of circulating FSH concentrations does not affect SPAP and consequently not GnSIF/AF bioactivity. Basal LH secretion and its synthesis were not significantly affected. Thus, these results may point to the absence of a regulatory role of FSH in GnRH-induced secretion of LH during a normal ovarian cycle.

At the same time, these injections had caused a substantial increase in GnRH-stimulated FSH release, which could be ascribed to an increased constitutive FSH synthesis and secretion (Chowdhurry et al., 1978; Jenner et al., 1982; Figures 2 and 3). According to this theory, inhibit blood concentrations were decreased but only after the multiple injections of anti-FSH. After the single injection protocols (1 and 2), FSH releases in vitro and pituitary FSH contents were already increased when inhibit blood concentrations were not significantly decreased. These measurements were carried out 3 or 5 days after the injections. It is possible, therefore, that a temporal decrease in inhibit concentrations has had sufficient impact on the gonadotrophs to increase pituitary FSH content and release for a few days.

From rat Sertoli cell conditioned media (Tio et al., 1994) and from porcine follicular fluid (Danforth and Cheng, 1995) two different GnSIF/AF proteins were purified and partly characterized. They differed from inhibit and follistatin. GnSIF/AF bioactivity has also been identified in human follicular fluid obtained from naturally cycling women (Koppenaal et al., 1992; Fowler et al., 1995) and in human follicular fluid and serum from FSH-stimulated women (Fowler et al., 1994; Balen et al., 1995; Byrne et al., 1995). Mroueh et al. (1996) demonstrated that porcine and human GnSIF/AF shared the same in-vitro bioactivity and chromatography and immunological characteristics.

If inhibit is defined as a hormone that inhibits constitutive FSH release, and GnSIF/AF as a factor that inhibits GnRH-induced LH (and FSH) release, some results show that both substances may have mutual intrinsic bioactivities (Campen and Vale, 1988; Farnsworth et al., 1988; Culler and Negro-Villar, 1989; Woodruff et al., 1993; Tio et al., 1994). Furthermore, immunoneutralization with inhibit antibodies increased the blood concentration of FSH with (Culler and Negro-Villar, 1989) or without (Rivier and Vale, 1989) that of LH, or prevented the decrease in LH concentrations after FSH treatment (Culler, 1992). These results taken together suggest that GnSIF/AF bioactivity may also be an intrinsic feature of inhibit.

If so, it is striking that the decreased inhibit concentrations and thus its intrinsic GnSIF/AF bioactivity did not affect GnRH-induced LH release. This may be due to the ED₅₀ of inhibit, more of which is needed to suppress the action of GnRH than to suppress FSH release (Tio et al., 1994; Balen et al., 1995; Byrne et al., 1995). Therefore, the most obvious conclusion is that under physiological conditions the intrinsic GnSIF/AF bioactivity of inhibit is not implicated in the control of LH release as its expression may be too low. When supraphysiological concentrations of inhibit are present, after stimulation with high dosages of FSH, the intrinsic GnSIF/AF activity of inhibit may be exposed sufficiently, leading to inhibitory effects on LH release. This view is strengthened by the observation of Culler (1992) who showed that immunoneutralization of inhibit prevented the GnSIF/AF mediated action of FSH.

Since anti-FSH treatment also decreases oestradiol concentrations, one might assume that changes in the oestradiol blood concentrations are responsible for the unchanged LH secretion patterns. However, if oestradiol had a potential GnSIF/AF action, its decrease would have caused an OVX-like effect. Moreover, oestradiol could be excluded as a GnSIF/AF (de Koning et al., 1980; Geiger et al., 1980; Koppenaal et al., 1991; Messinis and Templeton, 1991).

Serum LH concentrations were increased after the multiple anti-FSH injection protocols. When the pituitary glands of
these animals were tested in vitro, these increases were not accompanied by increased basal and GnRH-stimulated LH secretion rates. This might suggest that the increased blood LH concentrations are caused by an increased hypothalamic output of GnRH due to the diminished negative feedback by the decreased oestradiol blood concentrations. If the increase in GnRH release may be the consequence of decreased inhibin concentrations, it supports earlier observed actions of inhibin at the hypothalamic level (Lumpkin et al., 1983; Condon et al., 1983). Since the maximal pituitary LH responsiveness to GnRH in vitro is not affected, obviously, under these circumstances the GnSIF/AF bioactivity is sufficient to eliminate the potential effect of this increased GnRH release on the formation of SPAP.

To summarize, although FSH neutralization decreases inhibin and oestradiol, it does not affect GnSIF/AF bioactivity. The present results suggest, therefore, that during the major part of the rat ovarian cycle, inhibin does not contribute to the action of GnSIF/AF; and that GnSIF/AF release is not regulated by FSH. Further studies on the biological activity of GnSIF/AF, inhibin and follistatin on LH release are necessary. Also, the characterization of GnSIF/AF and the development of specific radioimmunoassays will be necessary for the further evaluation of a possible role for FSH in the release of GnSIF/AF.

Acknowledgements
The authors would like to thank Dr J.T.J. Uilenbroek (Erasmus University Rotterdam, the Netherlands) for supplying rabbit anti-ovine FSH serum and Prof. Dr F.H.de Jong (EUR) for the measurements of inhibin.

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FSH may not regulate GnSIF/AF release


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Received on March 3, 1998; accepted on July 22, 1998