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Review

Ovarian gonadotrophin surge-attenuating factor (GnSAF): where are we after 20 years of research?

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When gonadotrophin-stimulated IVF methods were being developed in the 1970s and 1980s, understanding of the physiology of FSH improved. In addition to its classic actions of stimulating aromatase activity and oestradiol secretion by ovarian granulosa cells, FSH was found to stimulate the ovarian production of an uncharacterized hormone known by its specific effect of reducing pituitary responsiveness to GnRH. This hormone has been called gonadotrophin surge-attenuating factor (GnSAF), gonadotropin surge-inhibiting factor (GnSIF), various abbreviations (GnSAF/IF, GnSIF/AF) and also attenuin. Although first described in the 1980s, GnSAF has still not been convincingly characterized and no published candidate amino acid sequences conclusively relate to GnSAF bioactivity. On the basis of superovulation studies and *in vitro* experimentation into the roles of steroids in regulating LH, GnRH and GnRH self-priming, the concept that GnSAF has a role in the regulation of LH secretion, the timing of the LH surge and the prevention of premature luteinization developed. For at least a decade, understanding of the specific GnSAF effects of reducing pituitary sensitivity to GnRH, especially GnRH self-priming and antagonizing the stimulatory effects of oestradiol on GnRH-induced LH secretion, supported this concept. However, improved knowledge of the changes in GnSAF bioactivity in follicular fluid and serum in women requires revision of this concept. The present authors propose that the main role of GnSAF is probably the negative regulation of pulsatile LH secretion, mainly during the first half of the follicular phase, indicating a critical role in the regulation of folliculogenesis and oestradiol secretion.

Evidence for an ovarian factor that reduced GnRH-induced LH secretion dates back to the late 1970s when de Jong *et al.* (1979) reported a < 10 kDa fraction of ethanol-extracted bovine follicular fluid (bFF) which reduced pituitary LH release. By the second half of the 1980s it was clear from the existing data that FSH treatment of women, monkeys and rats stimulated the production of an unidentified, non-steroidal factor. This factor was named gonadotrophin surge-inhibiting factor (GnSIF) or gonadotrophin surge-attenuating factor (GnSAF) (Ferraretti *et al.*, 1983; Littman and Hodgen, 1984; Sopelak and Hodgen, 1984; Messinis and Templeton, 1986). Schenken *et al.* (1984) showed that when monkeys were superovulated, serum collected from the ovarian vein inhibited the responsiveness of cultured rat pituitary cells to GnRH, the first clear evidence that GnSAF is an ovarian product. However, ovarian vein serum and follicular fluid normally contain high concentrations of sex steroids, especially oestradiol. Therefore, when trying to characterize this activity many researchers used charcoal extraction of a wide range of body fluids before bioassay for GnSAF using rat or sheep pituitary cells or perifused fragments. Thus GnSAF bioactivity has been detected in steroid-free serum and follicular fluid or ovarian extracts from superovulated and spontaneously cyclic women, cows, pigs and rats and from testis extract and Sertoli cell-conditioned medium (Fowler *et al.*, 1990, 1994a, 1995; Koppenaal, *et al.*, 1993; Kita *et al.*, 1994; Tio *et al.*, 1994; Danforth *et al.*, 1987; van Dieten *et al.*, 1999). Nevertheless, although GnSAF bioactivity could not be steroidal in nature, it remained to be demonstrated that the activity was not due to inhibin. Whereas inhibin had suppressive effects on GnRH-induced LH secretion at high concentrations in the rat pituitary (Farnworth *et al.*, 1988), this was not the case in the ovine pituitary where inhibin stimulated GnRH-induced LH secretion (Muttukrishna and Knight, 1990). Therefore, as steroid-free human follicular fluid inhibits GnRH-induced LH secretion from both rat and

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sheep pituitaries (Fowler *et al.*, 1994b), GnSAF cannot be due to inhibin. This conclusion is reinforced by the fact that co-incubation of human follicular fluid with inhibin antiserum has no effect on GnSAF bioactivity, as shown by continued reduction of GnRH-induced LH secretion, despite blocking the specific inhibin bioactivity of suppressing basal FSH secretion (Byrne *et al.*, 1995).

From the evidence for the existence of GnSAF it is clear that production of this hormone is regulated by FSH. The data supporting this are summarized in Fig. 1. Data obtained from *in vivo* studies after the administration of a single FSH injection to women (Fig. 1a) or multiple FSH injections to cows (Fig. 1b) as well as *in vitro* data based on granulosa cells from spontaneously cyclic women (Fig. 1c) are shown. GnSAF bioactivity in women (Messinis *et al.*, 1991, 1993a, 1994a) was calculated from measurements of circulating LH concentrations in response to GnRH injections, reduced LH secretion reflecting increased circulating GnSAF. GnSAF bioactivity in cows (Fowler and Price, 1997) was calculated by *in vitro* bioassay of serial blood samples. The bioassay depends upon the specific GnSAF effect of reducing GnRH-induced LH secretion, but not basal gonadotrophin release from cultured rat anterior pituitary cells. After the onset of FSH treatment in both women and cows, GnSAF bioactivity increased more rapidly than either oestradiol or inhibin. However, in post-menopausal women (Messinis *et al.*, 1994b) FSH treatment did not stimulate GnSAF activity. LH pulse amplitude and frequency were reduced after 20 h (Gosselin *et al.*, 2000) in FSH-treated cows, the former indicating increased GnSAF bioactivity *in vivo*. The stimulation of other candidate hormones for suppression of GnRH-induced LH secretion, that is oestradiol, inhibin A and inhibin B, was markedly slower than the stimulation of GnSAF (Messinis *et al.*, 1991, 1993a, 1994a; Fowler and Price, 1997; Burger *et al.*, 1998; Gosselin *et al.*, 2000; Welt *et al.*, 2001). Whether GnSAF acts on the hypothalamus directly, in this instance to affect GnRH pulse frequency, is unknown but is unlikely if GnSAF is about 60–70 kDa. However, by reducing LH pulse amplitude, GnSAF could reduce the number of apparent LH pulses detected.

The time course of stimulation of GnSAF bioactivity from the cultured human granulosa cells collected from small (6–9 mm) follicles was rapid (Fowler and Mason, 2000; Fig. 1c), supporting the observation of detectable GnSAF bioactivity *in vivo* within 8 h of a single FSH injection in women. It is noteworthy that the stimulation of GnSAF bioactivity *in vitro* occurred in the absence of androgen substrate for the granulosa cells, preventing these cells from manufacturing significant amounts of oestradiol in response to FSH treatment. Western blotting of the proteins secreted by these granulosa cells with a rat polyclonal antiserum with demonstrable GnSAF-blocking effects (Fowler *et al.*, 2002) showed upregulated expression of a number of proteins after 5 h exposure of the cells to FSH (Fowler and Mason, 2000). In a similar way, when medium conditioned by granulosa cells and theca and stromal tissues was added to a rat pituitary cell bioassay, GnSAF bioactivity was present only in granulosa cell-conditioned medium (Fowler *et al.*, 2002).

It is now clear that GnSAF bioactivity is not a result of inhibin A, inhibin B, oestradiol, progesterone or any other steroid hormone and is an FSH-stimulated ovarian product, originating specifically from the granulosa cell.

Current status of GnSAF characterization

A detailed examination of purification strategies for GnSAF is given in Fowler and Templeton (1996). Here this review will discuss the status of biochemical knowledge of GnSAF. To date there have been five main published attempts to characterize GnSAF, yielding amino acid sequences on four occasions, as shown in Table 1. Unfortunately, the collaborative purification attempt by the Danforth and Fowler groups (Mroueh *et al.*, 1996) obtained insufficient protein for Edman sequencing. This is one of the problems that has slowed progress on the identification of GnSAF: very small quantities of bioactive material after purification strategies. Despite the efforts made, not one of the sequences in Table 1 has been conclusively demonstrated as constituting the amino acid sequence for GnSAF bioactivity. Furthermore, when protein sequence matches have been taken from online databases, the candidate proteins do not match mass or isolectic point (pI) values obtained for GnSAF and are not known to have the ability to suppress GnRH-induced LH secretion (Fowler *et al.*, 2002).

The major problems with purification of GnSAF have been its co-elution with serum albumin and IgGs, and the fact that despite marked GnSAF bioactivity in a number of biological fluids, the protein appears to be present at low concentrations. The fact that the protein is present at low concentrations makes it difficult for sufficient bioactive material to be obtained for Edman sequencing. Indeed, several sequencing attempts have resulted in the identification of serum albumin and heavy chain IgGs despite numerous steps, such as blue dye affinity chromatography to reduce serum albumin contamination (P. A. Fowler, H. D. Mason, W. T. Melvin, B. Byrne, Y. Wilson, P. Cash, T. Sorsa-Leslie, L. Cowking, R. Bates and W. Harris, unpublished observations, 1990–1999).

The latest published purification procedure has sidestepped some of the problems previously encountered, by using a serum and BSA-free granulosa–luteal cell culture system (Fowler *et al.*, 2002). The authors found that human ovarian GnSAF bioactivity was associated with proteins of approximately 64 kDa and a pI of 5.7–5.8 pH. These values are in agreement with those of Danforth and Cheng (1995) and Mroueh *et al.* (1996), but are not in agreement with those of Tio *et al.* (1994),

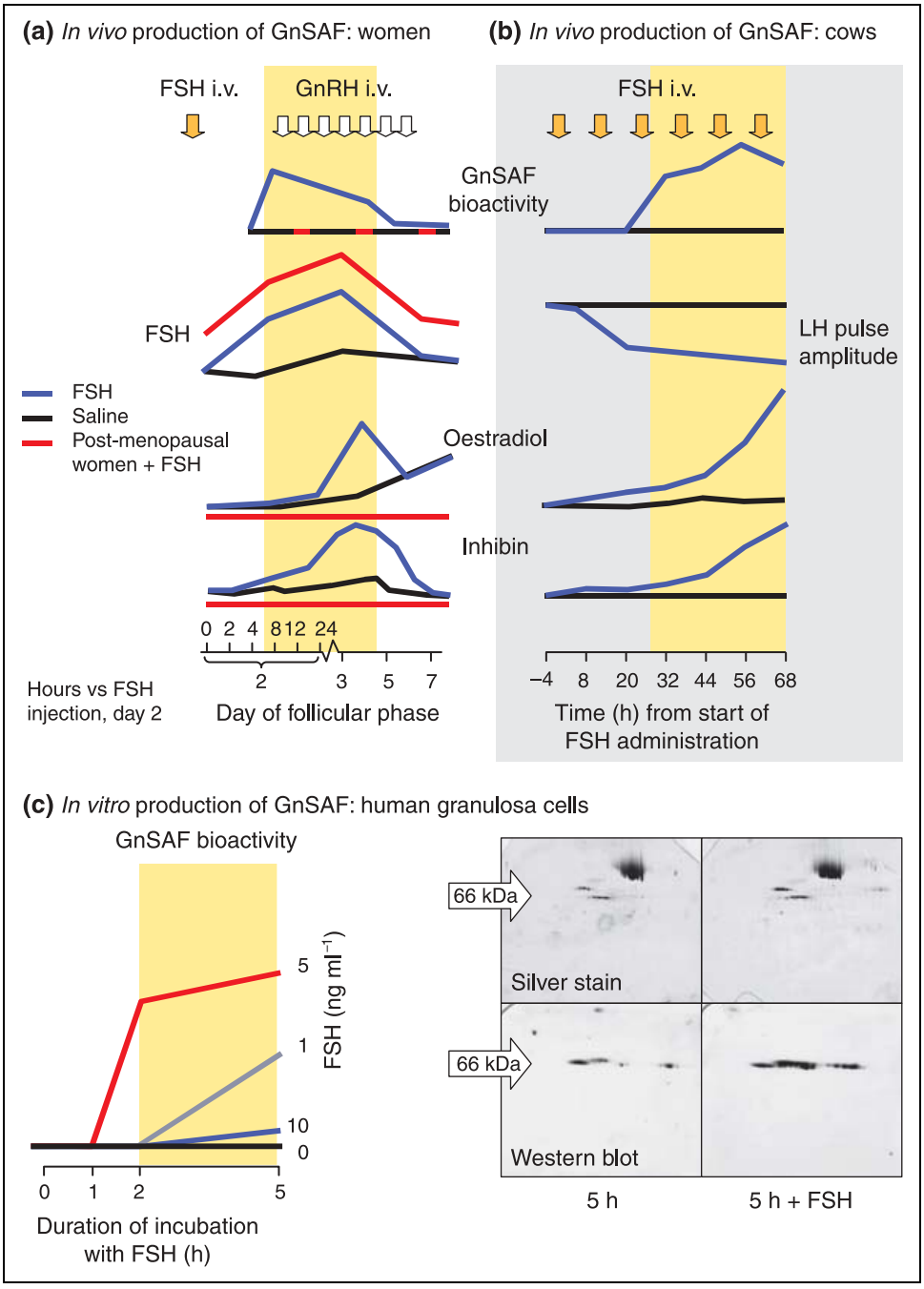

Fig. 1. The stimulation of gonadotrophin surge-attenuating factor (GnSAF) bioactivity by FSH both *in vivo* in (a) normally cyclic and post-menopausal women; (b) dairy cows; and (c) *in vitro* from granulosa cells collected from 6–9 mm follicles in spontaneously cyclic women. The periods of increased GnSAF are shown by vertical yellow bars. In women (a) day 2 of the follicular phase is subdivided (see italicized text above the horizontal axis) into hours relative to a single injection of FSH at 0 h, with GnRH challenges shown between day 2 and day 6 of the follicular phase. The days of the treatment period are shown in normal font below the horizontal axis. In cows (b) time points are shown relative to the first FSH injection at 0 h. FSH injections were repeated every 12 h. For the *in vitro* data (c) time points are shown relative to the addition of FSH to the culture wells at 0 h. Proteins secreted by the granulosa cells cultured *in vitro* in the presence and absence of FSH are shown in the form of silver-stained two-dimensional gels (upper gel pair) and western blot (lower gel pair) using a rat polyclonal antibody which blocks GnSAF bioactivity *in vitro* (Fowler *et al.*, 2002). Both show FSH-induced upregulation of protein at 60–70 kDa.

Table 1. Candidate gonadotrophin surge-attenuating factor (GnSAF) sequences

Molecular weight (kDa)	Amino acid sequence	Source	Species	Reference
37.0	NH_2: SDXXPQL No clear identification	Sertoli cell-conditioned medium	Rat	Tio *et al.*, 1994
69.0	NH_2: KPLAE No clear identification	Follicular fluid	Pig	Danforth and Cheng, 1995
63.0	? No clear identification	Superovulated follicular fluid	Human	Mroueh *et al.*, 1996
12.5	COOH:ALEVDETYVPK Identification: truncated C-terminus of serum albumin	Superovulated follicular fluid	Human	Pappa *et al.*, 1999
64.0	Internal: EPQVYVHAP No clear identification	Granulosa–luteal cell-conditioned medium	Human	Fowler *et al.*, 2002
64.0	NH_2: XVPQGNAXXN No clear identification			

or Pappa *et al.* (1999). Fowler *et al.* (2002) found that a 17 kDa fraction of human granulosa–luteal cell-conditioned medium caused minor reduction in GnRH-induced LH secretion from rat gonadotrophs, despite the main GnSAF bioactivity occurring at 64 kDa. This is similar to the 12.5 kDa activity reported by Pappa *et al.* (1999). However, in contrast to Pappa *et al.* (1999) who suggest that GnSAF is a truncated part of the C-terminus of serum albumin, Fowler and Danforth have not reported GnSAF bioactivity to be bound to serum albumin affinity chromatography steps. The activity reported at 37 kDa by Tio *et al.* (1994) used conditioned medium from Sertoli cells rather than granulosa–luteal cells. Again in contrast to findings by other groups (Danforth and Cheng, 1995; Mroueh *et al.*, 1996; Pappa *et al.*, 1999; Fowler *et al.*, 2002), purified material prepared by Tio *et al.* (1994) retained inhibin-like bioactivity as it caused a reduction in basal FSH secretion from rat pituitary cultures, indicating that the bioactivity is probably not GnSAF, but possibly a male homologue.

The internal and N-terminal amino acid sequences reported by Fowler *et al.* (2002) may be part of the same protein, but neither matches the other published putative GnSAF sequences (Tio *et al.*, 1994; Danforth and Cheng, 1995; Pappa *et al.*, 1999). Furthermore, no common proteins yet identified contain combinations of the five candidate GnSAF amino acid sequences. However, it is entirely possible that when GnSAF is finally convincingly sequenced, one or more of these putative sequences may prove to have been part of the bioactive molecule.

The current GnSAF purification strategy in our research group involves the use of phage display antibody libraries to circumvent the problems encountered by all groups attempting to purify GnSAF by conventional means. Sorsa-Leslie *et al.* (2001, 2002) have successfully used phage display techniques to produce antibodies against partially purified human granulosa–luteal cell-conditioned medium. Three of these antibodies were found to block the effects of human GnSAF on GnRH-induced LH secretion from rat pituitary cell cultures. The phage display-derived antibodies also appear to be suitable for immunopurification of GnSAF and have been engineered into human IgGs in order to scale-up the immunopurification process. Both the phage antibodies (Sorsa-Leslie *et al.*, 2003) and the anti-GnSAF rat polyclonal antiserum reported by Fowler *et al.* (2002) have been used for the successful immunopurification of GnSAF bioactivity and the purified preparations are currently being used for sequencing experiments. It may be therefore that GnSAF is on the point of being convincingly characterized.

GnSAF in women

GnSAF in the follicle

The production of GnSAF in the ovarian follicle is clearly related to follicle size in both stimulated and spontaneous cycles, and follicular fluid from follicles < 11 mm (stimulated cycles, Fowler *et al.*, 1994b) or 6–8 mm (spontaneous cycles, Fowler *et al.*, 2001) contain the greatest amount of GnSAF bioactivity. This is shown in Fig. 2, which clearly demonstrates a lack of either positive or negative correlation between follicular fluid GnSAF bioactivity and follicular fluid concentrations of inhibin A, inhibin B, oestradiol or progesterone (McNatty, 1981; Westergaard *et al.*, 1986; Fowler *et al.*, 1994b, 2001; Magoffin and Jakimuik, 1997). The same holds true for activin (Fowler *et al.*, 2001). In a similar way, small follicles in pig ovaries contain the highest concentrations of GnSAF, with bioactivity falling sharply in preovulatory follicles in particular (Kita *et al.*, 1994). These findings demonstrate that GnSAF is primarily produced by small growing follicles.

GnSAF in the circulation

An important clue to the physiological roles of many hormones is the pattern of changes in their concentration in the peripheral circulation. For GnSAF, certain changes in serum concentrations, such as a fall before the LH surge, would be essential to support the hypothesis that

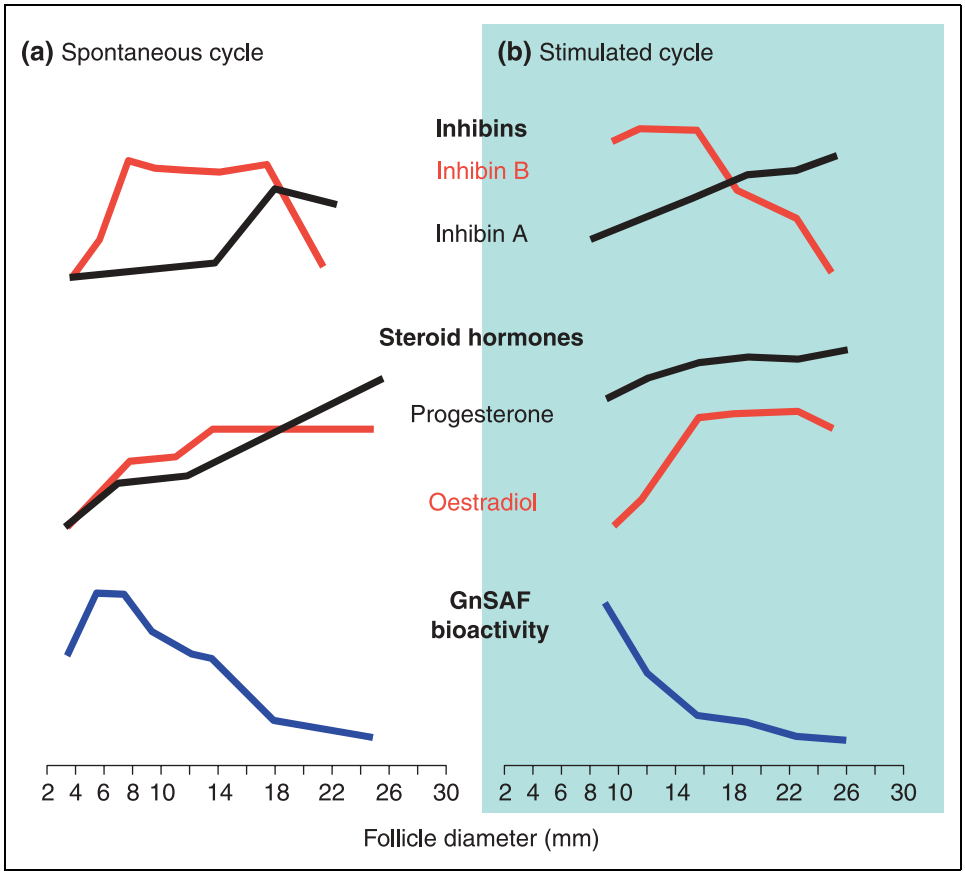

Fig. 2. Human follicular fluid gonadotrophin surge-attenuating factor (GnSAF) bioactivity is greatest in small follicles whether collected from (a) spontaneous or (b) stimulated cycles. In both types of cycle follicular fluid GnSAF content is related to follicle size, with GnSAF production decreasing with increasing follicle diameter. Note that concentrations of steroid hormones and the inhibins in follicular fluid are known to be extremely variable and only general trends are shown in this diagram.

it has a role in the regulation of the timing and magnitude of the LH surge. However, whereas GnSAF bioactivity is readily measurable in serum from women undergoing ovarian stimulation for IVF, the detection of GnSAF in serum from spontaneously cyclic women has proved more difficult. It is predictable that as FSH-stimulated women have reduced responsiveness to GnRH, their circulating GnSAF titres would be higher than those of untreated women. Nevertheless, this slowed attempts to determine the GnSAF concentrations in the serum of spontaneously cyclic women throughout the menstrual cycle. Byrne *et al.* (1993) concentrated serum from spontaneously cyclic women tenfold before bioassay and found that GnSAF bioactivity was low early in the follicular phase, peaked in the mid-follicular phase and then declined in the late follicular phase.

Improvements to the GnSAF bioassay developed by our group allowed the detection of GnSAF bioactivity in unprocessed serum from spontaneously cyclic women (Martinez *et al.*, 2002). These data, combined with the data from Byrne *et al.* (1993) are shown in Fig. 3. As expected, GnSAF decreases as the number of small follicles declines after follicular dominance is established. The corpus luteum does not appear to produce GnSAF in women, but small developing follicles may produce GnSAF during the luteal phase (Messinis *et al.*, 1996). Furthermore, decreasing pituitary responsiveness to GnRH during the luteal–follicular transition indicates that small follicles, responding to the luteal–follicular FSH rise, may be producing GnSAF bioactivity at the end of the luteal phase (Messinis *et al.*, 1993b). Certainly, by very early in the follicular phase GnSAF bioactivity is high in the circulation (Martinez *et al.*, 2002). Overall, the circulating profiles of inhibin A, inhibin B, oestradiol and progesterone, derived from a number of studies including Groome *et al.* (1996), do not explain the *in vivo* and *in vitro* bioassay data upon which the proposed pattern of GnSAF concentrations in the peripheral circulation of women is based.

The emergence of new data supporting earlier suggestions of the occurrence of follicular waves in women during the follicular phase (Baerwald *et al.*, 2003a,b)

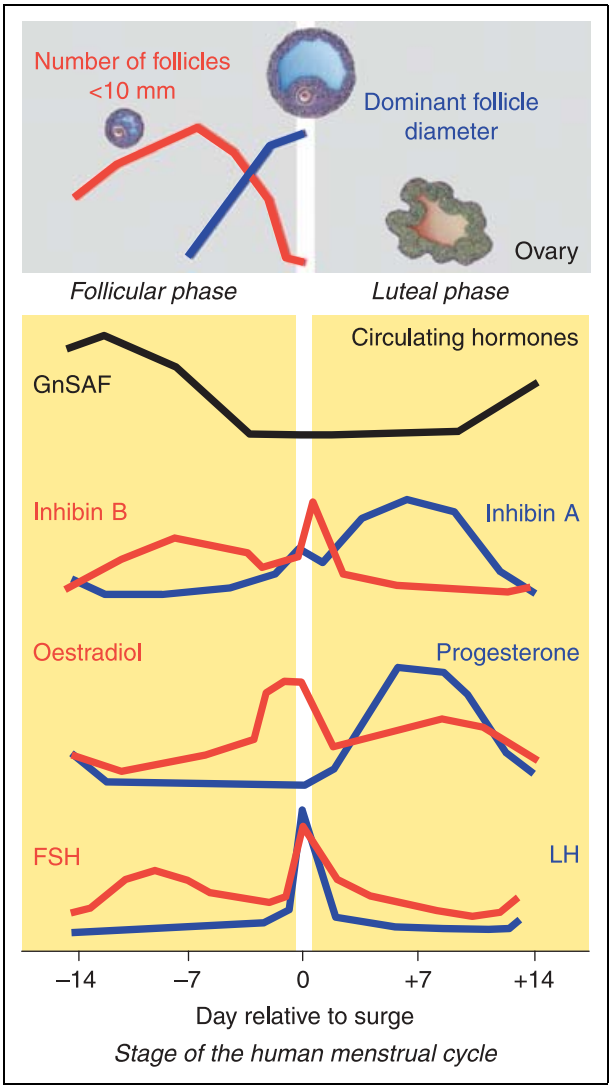

Fig. 3. Gonadotrophin surge-attenuating factor (GnSAF) in relation to the menstrual cycle. Data on GnSAF bioactivity is predominantly derived from studies based on the present authors' *in vitro* rat pituitary cell bioassay for GnSAF using serum from spontaneously cyclic women, and on *in vivo* studies of LH responses to GnRH in women. Circulating GnSAF peaks during the early–mid-follicular phase, is low during the mid–late follicular and most of the luteal phases, probably increasing with renewed follicular development driven by the inter-cycle FSH rise.

has the implication that the pattern of circulating GnSAF concentrations presented in this review may be subject to at least some modification once a more sensitive means of GnSAF detection, such as an immunoassay, has been developed.

A role for GnSAF in the endocrine regulation of the menstrual cycle?

In women, the cyclic changes in gonadotrophins drive ovarian function and are regulated by feedback from the ovary on the hypothalamus–pituitary functional unit (reviewed by Chabbert Buffet *et al.*, 1998).

Inter-cycle FSH rise

For much of the luteal phase the hypothalamus and pituitary are 'clamped' in a state of reduced activity as far as gonadotrophin secretion is concerned. However, towards the end of the luteal phase the function of the corpus luteum deteriorates, oestradiol, progesterone and inhibin A begin to decrease and the pituitary undergoes a rebound, resulting in the inter-cycle FSH rise. The signal drives recruitment of a cohort of small follicles which undergo a final development phase.

Oestradiol and inhibin B

As they grow, the small follicles secrete increasing quantities of inhibin B, peaking at about day 8 of the follicular phase. In a similar way, early in the follicular phase the growing follicles produce small quantities of oestradiol, which has a negative feedback effect on LH secretion via hypothalamic sites of action. The inhibin B has a negative feedback effect on FSH which then begins to decrease. Whereas there are ample data indicating that oestradiol suppresses FSH secretion (for example Bassett and Zeleznik, 1990), recent studies indicate that in women the inhibins play a major role in the suppression of FSH as the follicular phase progresses (Welt *et al.*, 2003). In these studies the importance of oestradiol in the negative regulation of FSH is mainly via a hypothalamic action. However, several studies, as reviewed by Zeleznik and Benyo (1994), support the concept that oestradiol is a significant factor regulating the reduction in circulating FSH during the mid–late follicular phase.

Whether due to inhibin B or oestradiol, FSH decreases and only the dominant follicle survives the loss of FSH support and this undergoes explosive growth, supported by the low circulating titres of LH (Sullivan *et al.*, 1999). It is important that during the follicular phase inhibin B production by smaller follicles is primarily stimulated by FSH whereas LH stimulates inhibin A production from more mature follicles (Welt *et al.*, 2001).

As the follicles grow, oestradiol titres increase and oestradiol secretion from the dominant follicle increases exponentially in the last few days of the follicular phase. During this stage, the feedback effect of oestradiol switches from negative to positive, acting as a powerful signal to both hypothalamus and pituitary to produce the GnRH and LH surges.

GnRH pulses, the GnRH surge and GnRH self-priming

Unlike in many other species, including non-human primates (Pau *et al.*, 1993), there is no direct evidence for a preovulatory GnRH surge in women. A recent review

(Park *et al.*, 2002) assessed the evidence from a number of studies during the 1970s and 1980s for increased GnRH pulse frequency during the periovulatory period, on the basis of LH pulses. However, there is also experimental evidence that GnRH is reduced at the time of the human LH surge (Martin *et al.*, 1998). The role of progesterone should not be ignored and McCartney *et al.* (2002) indicated that increasing GnRH pulse frequency in the course of the follicular phase partly reflects the declining effects of negative feedback actions of luteal phase progesterone, a conclusion supported by Skinner *et al.* (2000). Whether or not there is a periovulatory GnRH surge in humans, the patterns of hypothalamic GnRH production are known to be important in the regulation of gonadotrophin secretion because abnormal GnRH pulse patterns are associated with a range of reproductive dysfunctions (Marshall *et al.*, 2001). If GnRH pulses are sufficiently frequent, especially after a few days of increased oestradiol, then GnRH self-priming occurs. Self-priming, or self-potentiation, is the phenomenon whereby a subsequent GnRH pulse stimulates a larger LH pulse from the pituitary than the preceding GnRH pulse: the pituitary GnRH receptors become 'primed'. The exact role of self-priming in the generation of the LH surge is debatable. In a recent review, de Koning *et al.* (2001) concluded that low or absent negative feedback by GnSAF contributed to increased LH secretion during the periovulatory period rather than this being due to increased GnRH effects via self-priming.

The puzzle

The switching of oestradiol feedback from negative to positive and back to negative during the menstrual cycle and the fact that overt GnRH self-priming is limited to the periovulatory period remain incompletely understood. If steroid hormones alone are responsible for the LH surge, then reproducing mid-cycle steroid concentrations in women or monkeys should result in a normal LH surge. Characteristically, the LH surge that results is reduced in amplitude but nevertheless very clear (Yamaji *et al.*, 1971; Taylor *et al.*, 1995). However, these studies involve either (1) ovariectomized monkeys which would therefore have no circulating GnSAF; or (2) women administered late follicular phase steroids during the early–mid follicular phase. In this case the secretion of LH (82.7 iu l^{-1} with oestradiol alone and 69.7 iu l^{-1} with oestradiol and progesterone versus 121.7 iu l^{-1} in normal controls) is clearly reduced but not abolished. This effect is probably due to the fact that higher (mid-follicular phase) concentrations of GnSAF would be present than would be seen during the normal late follicular phase. Therefore, the result would be that sufficient GnSAF would be present to antagonize the effects of the steroids. However, in studies in non-ovariectomized monkeys (Karsch *et al.*, 1973), the administration of high concentrations of oestradiol, similar to those seen in periovulatory animals, stimulated LH surges in half the monkeys after 36 h exposure in the early follicular phase. It may be that during this 36 h period the exogenous oestradiol reduces FSH concentration and the population of small follicles exhibits a declining production of GnSAF. Oestradiol could then overcome the inhibitory effects of GnSAF, a possibility highlighted by the way in which supraphysiological concentrations of oestradiol induced LH surges after only 24 h exposure.

In summary, overall these data indicate that steroids alone do not explain the control of the LH surge although high concentrations of oestradiol stimulate the occurrence of the LH surge. However, the fact that GnSAF bioactivity has been observed below the level of detection in the present authors' bioassay in women during the late follicular phase (Martinez *et al.*, 2002) indicates that there would be very little or no GnSAF to antagonize oestradiol-positive feedback or GnRH self-priming during the periovulatory period. However, a study in sheep (Clarke, 1995) concluded that the increase in pituitary responsiveness to GnRH at the time of the LH surge was caused by the disappearance of an inhibitory signal that was not steroidal in nature, and which preceded any increase in GnRH release. This primary role of oestrogen in stimulating the LH surge has been a feature of reviews on this topic for some time (for example Shoham *et al.*, 1995) and is based on work such as that by Knobil's group (Ordog *et al.*, 1998) who have shown that oestradiol has undoubted positive feedback effects on LH secretion, rather than merely negative effects. However, even in post-menopausal women, exogenous oestradiol and progesterone retained some negative feedback effects on LH secretion (Gill *et al.*, 2002). It was unfortunate that the authors did not also perform GnRH challenges. Given the well-known critical functions of steroids in regulating the LH surge, an interesting possibility is a possible role for GnSAF in regulating follicular steroidogenesis by reducing LH pulse amplitude. In cows for instance, increased LH and subsequently oestradiol is associated with subsequent follicular deviation to dominance (reviewed by Ginther *et al.*, 2001). Therefore, rather than regulating the timing of the LH surge by directly reducing pituitary responsiveness to GnRH, GnSAF may have a role in regulating the timing of the LH surge by regulating follicular phase LH pulse amplitude and therefore playing a role in the control of oestradiol secretion.

Whereas oestradiol potentiates the suppressive effects of GnSAF on GnRH-induced LH secretion (P. A. Fowler, H. D. Mason, Y. Wilson, L. Cowking, T. A. Bramley and B. Byrne, unpublished; Tijssen *et al.*, 1997, van Dieten *et al.*, 1999), it is not clear whether such effects are evident *in vivo* in women and this may be an artefactual effect of study design. There is also extensive *in vitro* and *in vivo* evidence for the suppressive effects of GnSAF on GnRH self-priming (Messinis and Templeton, 1991;

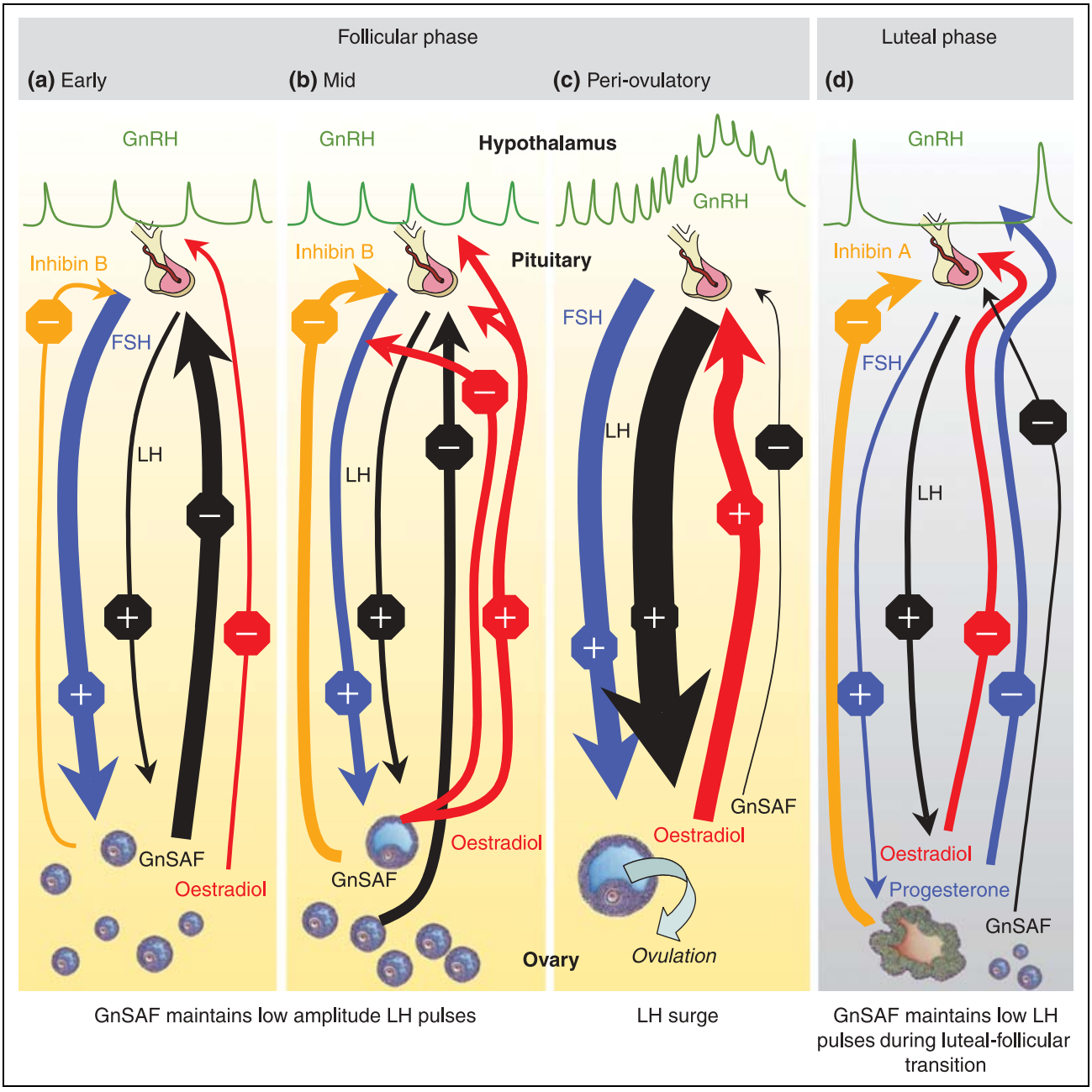

Fig. 4. Outline of the endocrine regulation of the menstrual cycle. The thickness of arrows is indicative of circulating hormone concentrations and their relative effect, as indicated by + (positive) or – (negative) signs. (a) Early follicular phase: FSH drives follicular development, with oestradiol and high concentrations of gonadotrophin surge-attenuating factor (GnSAF) exerting negative feedback on the amplitude of LH pulses, driven by GnRH pulses at a rate > 1 per h. (b) Mid-follicular phase: inhibin B or oestradiol inhibit FSH release, leading to selection of the dominant follicle. As the subordinate follicles become atretic GnSAF begins to decrease from peak concentrations although GnSAF continues to moderate LH pulse amplitude and therefore oestradiol production. GnRH pulses are approximately circhoral. (c) Periovulatory period: high concentrations of oestradiol, stimulating high frequency GnRH pulses (up to 1 pulse every 15 min) easily overcome negligible GnSAF antagonism. Prolonged oestradiol positive feedback in the presence of a small increase in progesterone may lead to a GnRH surge (as occurs in non-human primates), events leading to the LH surge and ovulation. (d) Luteal phase: the hypothalamus–pituitary–ovary axis is clamped with negative feedback from oestradiol, progesterone and inhibin A produced by the corpus luteum. GnRH pulse frequency decreases to approximately one pulse every 4 h, whereas LH pulse amplitude is initially high. Low concentrations of GnSAF are released from quiescent small follicles. At the end of the luteal phase the inter-cycle FSH increase begins the next cycle of folliculogenesis and GnSAF production increases as a new cohort of small follicles begin to grow rapidly. During the luteal–follicular transition GnSAF may reduce LH pulse amplitude as GnRH pulse frequency increases.

Koppenaal *et al.*, 1992, 1993; Fowler *et al.*, 1994b; Byrne *et al.*, 1996). This effect is not due to occupancy of the pituitary GnRH receptor, but involves blockade of several second messenger mechanisms (Fowler *et al.*, 1994c; Tijssen *et al.*, 1997). A preliminary study indicates that the as yet unidentified GnSAF receptor may act via the c-AMP signal transduction pathway (Helder *et al.*, 1997). Therefore, it may be hypothesized that GnSAF will antagonize the effects of GnRH on the pituitary by interfering with the positive feedback effects of oestradiol and reducing post-GnRH receptor second messenger signalling to prevent GnRH self-priming and reduce the resulting amplitude of LH pulses.

A working hypothesis for the role of GnSAF in regulating LH secretion

A schematic diagram for the potential role of GnSAF in the endocrine regulation of the menstrual cycle and LH surge is shown in Fig. 4. Early in the follicular phase (Fig. 4a), small growing follicles, stimulated by FSH, produce high concentrations of GnSAF and small quantities of oestradiol, the GnSAF in particular having a negative feedback effect on pituitary responsiveness to GnRH and thus maintaining low concentrations of LH and keeping LH pulse amplitude low. As the follicles grow, inhibin B secretion peaks during the mid-follicular phase (Fig. 4b). Inhibin B or oestradiol cause FSH secretion to decline, resulting in the selection and further growth of the dominant follicle, but atresia of subordinate follicles. At this stage oestradiol begins to switch to positive feedback, but this is antagonized to an extent by continuing GnSAF production, resulting in the maintenance of low concentrations of LH and small LH pulses. Nevertheless, increasing GnRH pulse frequency is occurring, slowly rising from the roughly circhoral pulses of the early–mid follicular phase. Explosive dominant follicle growth into the preovulatory period (Fig. 4c) is matched by a large increase in circulating oestradiol and atresia of the subordinate follicles, resulting in a decrease in GnSAF production. At this time falling GnSAF may act permissively in terms of follicular steroidogenesis by allowing higher LH pulse amplitude. The oestradiol also has hypothalamic effects, stimulating GnRH neurones (Herbison, 1998). The positive feedback effects of oestradiol on the hypothalamus and pituitary become predominant and rapid GnRH pulses, possibly augmented by GnRH self-priming or even a GnRH surge, occur. There is very little, if any, GnSAF remaining in the circulation by this stage to inhibit pituitary responsiveness to GnRH and the LH surge results, followed by ovulation some 36 h later. During the luteal phase (Fig. 4d), the corpus luteum maintains pituitary 'clamping' via progesterone and inhibin A signalling and the next cohort of small follicles is relatively quiescent. The role of GnSAF in regulating LH secretion is probably negligible at this time. However, towards the end of the luteal phase, failure of the corpus luteum and the resulting inter-cycle FSH increase stimulates follicular growth and GnSAF production. The latter will again begin to inhibit LH pulse amplitude, allowing a more favourable environment for follicular development.

It must be noted that this tentative scheme requires validation by carefully designed studies once the GnSAF molecule has been fully characterized.

Conclusions

GnSAF is a non-steroidal, non-inhibin, ovarian hormone, secreted by the granulosa cell in response to FSH. It has the specific effect of negatively regulating the release of GnRH-induced LH, therefore having a probable role in the regulation of the magnitude of LH pulses, co-ordinating the LH signal with follicular development and steroidogenesis in women and other mammals. In terms of the timing and magnitude of the preovulatory LH surge, GnSAF probably has far less direct effect than previously thought. The study of the role and importance of GnSAF has been hampered by the extreme difficulty experienced in definitively characterizing the bioactive molecule. Nevertheless, substantial progress in identifying the nature of GnSAF and its putative role in the regulation of the LH surge has been made in the last 20 years. Furthermore, recent progress by our group indicates that GnSAF may soon be identified. This would resolve problems associated with the absence of an immunoassay to measure circulating and follicular fluid GnSAF titres and the lack of homogeneous GnSAF preparations for physiological investigation. It is hoped that exciting studies to establish the importance and potential application of GnSAF in the regulation of reproduction in the female will be possible in the near future.

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