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Attenuin: What It Is, How It Works and What It Does

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1. Introduction

The reproductive function in female mammals, unlike that of males, is cyclical in nature. Whilst testosterone exerts a wholly negative control, regulation of the ovarian cycle is effected through a complex series of positive and negative feedback mechanisms involving the ovary, the pituitary and the hypothalamus. Although the role of these feedback mechanisms has been known for many years, our knowledge of the detailed workings involved continues to expand. Reproductive-axis hormones have proved to be similar in all the mammalian species studied to date. The female cycle can generally be divided into the follicular, periovulatory (preovulatory and ovulatory) and luteal phases. The pituitary hormones – the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) – remain at basal levels throughout most of the reproductive cycle, due to negative regulation by ovarian hormones (mainly estradiol and progesterone; see fig. 1 for additional details). The physiological capacity of steroids to control gonadotropin secretion is evident in a marked increase in plasma LH and FSH levels after the menopause or following ovariectomy. During the preovulatory phase, the estradiol negative feedback switches to positive. Major positive actions by estrogens include: a 20- to 50-fold increase in pituitary sensitivity to gonadotropin-releasing hormone (GnRH) (Speight et al., 1981); an increase in pituitary GnRH receptors, in concert with GnRH (Clayton et al., 1980); an increase in progesterone receptors (PR) in both pituitary and hypothalamus (Conneely et al., 1989); stimulation of GnRH synthesis and release, resulting in preovulatory GnRH secretion (Sarkar et al., 1976); and a drop in GnRH metabolism by pituitary cells (Danforth et al., 1990). These positive actions of estradiol lead to the appearance of GnRH self-priming. GnRH self-priming is an event with a twofold effect: it prompts an exponential increase in pituitary responsiveness to GnRH, and it coordinates increased responsiveness with enhanced GnRH release, ensuring that both occur at the same time and thus guaranteeing preovulatory LH secretion (Fink, 1995). In rats, this self-priming effect has been found to be more effective when GnRH pulses occur at hourly intervals (Fink, 1995), a frequency also reported to be optimal in monkeys with hypothalamic lesions (Knobil, 1980) and in women with idiopathic hypogonadotropic hypogonadism (Crowley et al., 1985). How does GnRH come to possess this unique property? Probably because the preovulatory LH surge is indispensable for species reproduction, and the fact that a very small amount of GnRH induces a disproportionate release of LH makes the process both economic and efficient

(Fink, 1995). In conclusion, although the cyclical nature of reproduction has been recognized in both humans and animals for centuries, the sequence of events involved has only recently become clear, and a number of questions remain unanswered (Schwartz, 2000).

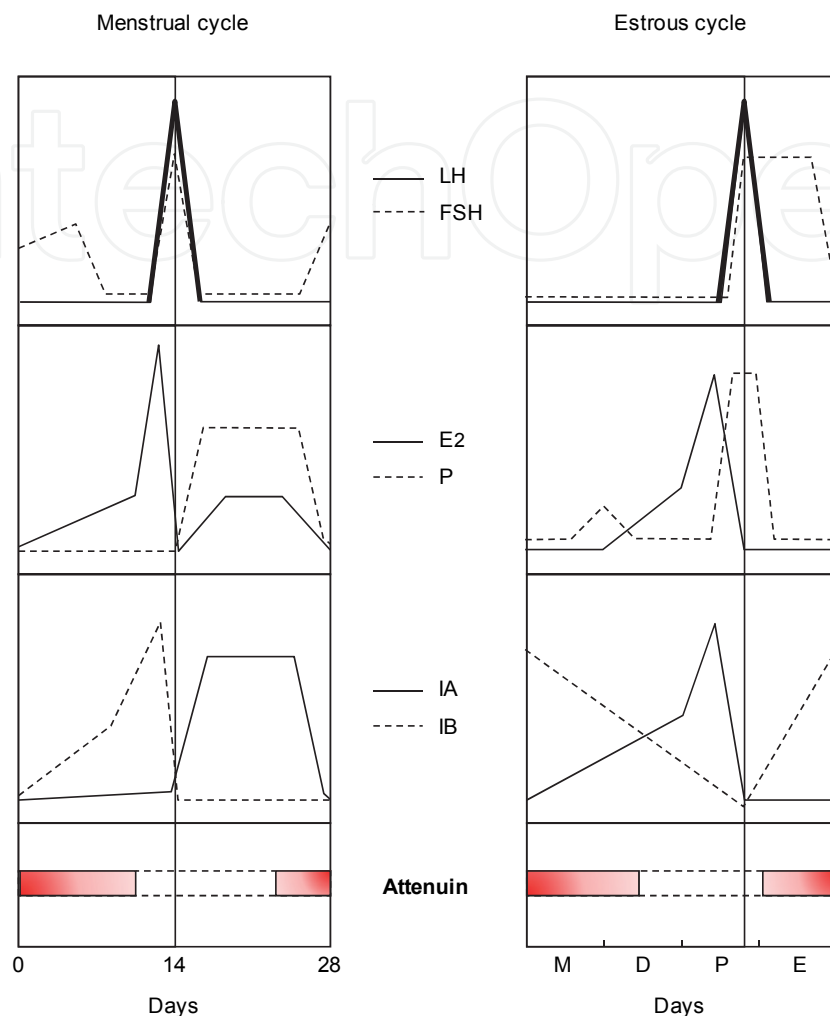


Fig. 1. Diagram showing pituitary and ovarian hormone fluctuation during menstrual (woman) and estrous (rat) cycles. LH: luteinizing hormone; FSH: follicle-stimulating hormone; E2: estradiol; P: progesterone; IA: inhibin A; IB: inhibin B; M: metestrus; D: diestrus; P: proestrus; E: estrus. Days of cycle are synchronized with the LH surge (day 14 in women and proestrous afternoon in rats). During the menstrual cycle, and shortly after functional luteolysis (drop in E2, P and IA) the secretion of FSH increased, stimulating the ovaries and inducing follicular recruitment. In the rat, secondary FSH secretion during early estrus stimulated the ovaries, similarly inducing follicular recruitment. In both cases, increased FSH secretion augmented attenuin bioactivity by stimulating the ovaries (lower panels). Attenuin bioactivity prevented premature occurrence of the LH surge (see text for additional details).

One crucial and still-unresolved question is how the negative feedback exerted by estradiol suddenly switches to positive, resulting in the preovulatory LH surge. Though this issue has been argued back and forth, no agreement has yet been reached. The positive effects of estradiol do not all occur at the same time, nor are they present only during preovulatory

secretion. For example, the increased steroid-induced pituitary response to GnRH is directly linked to steroid levels during the follicular phase (Yen et al., 1972). Moreover, GnRH self-priming can be observed experimentally long before the LH surge is due (Waring & Turgeon, 1980). All this would suggest that steroids have to reach a critical threshold before generating any LH surge (de Koning, 1995), or alternatively that some factor is exerting a “braking” effect on the estradiol positive feedback (Whitehead, 1990). Studies of *in vitro* fertilization (IVF) methods argue against the idea of a critical level of estradiol (200 pg/ml during 36–48 h): reports indicate that in FSH-treated women with multiple follicles able to produce supraphysiological levels of estradiol from the beginning of the follicular phase, preovulatory LH secretion does not occur prematurely, and when it does appear it is diminished (Ferraretti et al., 1983; Glasier et al., 1988; Messinis et al., 1986). High levels of steroids are unlikely to inhibit the preovulatory LH surge, and high exogenous doses of estradiol are unable to suppress LH secretion in normal menstrual cycles (Messinis & Templeton, 1987). There is thus no evidence to support the hypothesis that abnormal estradiol levels inhibit preovulatory LH secretion in stimulated cycles; indeed, the evidence so far points to the existence of some non-steroidal factor, produced by stimulated ovaries and able to suppress steroidal positive feedback. Over recent decades, the search for putative new substances secreted by the ovaries has provided new insights into the role of the ovaries in gonadotropin secretion. This chapter focuses on the factor known as attenuin (Messinis & Templeton, 1989; Sopelak & Hodgen, 1984).

1.1 Evidence for the existence of attenuin

The development of IVF methods for addressing infertility in the 1970s and 1980s also shed new light on the role of FSH. In addition to its classic functions (stimulation of aromatase activity and estradiol secretion in granulosa cells, and participation in follicular selection mechanisms), FSH was found to stimulate the production of an ovarian factor with a special ability to reduce preovulatory LH secretion in women. The first evidence for an ovarian factor that attenuated GnRH-induced LH secretion appeared in the late 70s, when de Jong et al. (1979), using bovine follicular fluid (bFF), observed a drop in the responsiveness of rat pituitary cells to GnRH. Over the following decade, a number of studies pointed to the existence of a non-steroidal ovarian factor that reduced preovulatory LH release when FSH was administered during the follicular phase in women (Ferraretti et al., 1983; Messinis & Templeton, 1986), monkeys (Littman & Hodgen, 1984; Schenken et al., 1984) and rats (Busbridge et al., 1988; Geiger et al., 1980). This factor was called gonadotropin surge-attenuating factor (GnSAF), gonadotropin surge-inhibiting factor (GnSIF) or simply attenuin. In 1984, Schenken et al. demonstrated for the first time a direct effect of FSH on gonadotropin secretion, involving one or more ovarian factor(s). Ovarian venous serum (OVS) was collected from FSH-treated monkeys before and after aspiration of the right ovarian follicle; the left ovarian follicle remained intact. Serum from right ovaries before aspirated and from intact left ovaries inhibited GnRH responsiveness in rat pituitary cultures, suggesting that exogenous FSH increased OVS concentrations of a non-steroidal ovarian factor with gonadotropin-inhibiting activity. The ovarian origin of attenuin was confirmed when Fowler et al. (2002), culturing theca, stroma and granulosa cells from cyclic women, showed that attenuin bioactivity was present only in granulosa-cell-conditioned medium. Use of a superovulated protocol as a model for increased attenuin bioactivity posed a number of problems, including elevated peripheral estradiol and inhibin levels during gonadotropin administration (de Jong, 1988; Messinis & Templeton, 1988;

Muttukrishna et al., 1994), which raised doubts as to the origin of this bioactivity. With regard to estradiol, studies carried out in women have proved that effects on LH secretion are not due to negative feedback: Messinis et al. (1991, 1993) demonstrated that a single FSH injection had dose-dependent suppressive effects on pituitary responsiveness to exogenous GnRH pulses. This effect was seen as early as 8 h after FSH administration, although circulating estradiol did not increase significantly until at least 24 h after gonadotropin administration. Subsequently, this group also concluded that attenuin bioactivity in FSH-treated women could not be due to an increase in circulating total α -inhibin (Messinis et al., 1991, 1993, 1994, 1996).

Attenuin bioactivity has so far been found in serum, FF and ovarian extracts from superovulated (Fowler et al., 1994a) and cyclic women (Fowler et al., 1995a), in bFF (Van Dieten et al., 1999), in porcine FF (pFF; Danforth et al., 1987; Kita et al., 1994), in rat ovarian extracts (de Koning et al., 1989) and in rat testicular extracts (Tio et al., 1994). It should be stressed that attenuin bioactivity has been observed in human FF (hFF) from women during an untreated spontaneous cycle, suggesting that it plays a physiological role as a central regulator (Busbridge et al., 1990; Byrne et al., 1993).

1.2 Current status of attenuin characterization

In 1987 Danforth et al., using pFF, showed that attenuin bioactivity displayed certain characteristics: it was resistant to moderate heating (60 °C for 60 min), fully recoverable from an acetone precipitation, and not bound by a heparin/sepharose affinity matrix of the sort widely used to isolate inhibin. To date, attempts to characterize attenuin have proved relatively unsuccessful. Purification strategies have obtained very small amounts of bioactive material and an abundance of different proteins. The fact that the protein is present at very low concentrations makes it difficult to use Edman sequencing, leading to a whole range of different results. Tio et al. (1994) isolated inhibin and attenuin by high performance liquid chromatography from 32 liters of rat Sertoli cell-conditioned medium. Both peptides were separated on polyacrylamide gel; however the partial N-terminal sequence of the 37 kDa protein with attenuin bioactivity did not match gene and protein databases (Genbank and PIR). Subsequently, employing an elegant sequential series of purification techniques, Danforth & Cheng (1995) found a 69 kDa monomeric polypeptide in pFF that inhibited GnRH-stimulated LH secretion, but partial N-terminal analysis showed no homology with other reproductive hormones. Mroueh et al. (1996) also found a protein with attenuin bioactivity in hFF, similar to that reported in pFF (63 kDa). However, Pappa et al. (1999), starting from the same source (hFF) treated with multiple and complex purification techniques, described a protein with a molecular mass of 12.5 kDa. This protein was identified by mass spectrometry as a truncated part of the C-terminus of human serum albumin (HSA). The next published purification procedure side-stepped serum albumin contamination problems by using a serum- and BSA-free granulosa-luteal cell culture system (Fowler et al., 2002). The isolated attenuin-bioactivity protein had a molecular weight of 60-70 kDa and an isoelectric point (pI) of 5.7-5.8 pH. The internal and N-terminal amino acid sequences did not display significant homology with other accessions in the protein-sequence database. Attenuin is therefore thought to be a peptide, different from inhibin, with a postulated molecular weight which varies from less than 37 kDa (Tio et al., 1994) to 64-69 kDa (Danforth & Cheng, 1995; Fowler et al., 2002). These differences may reflect the presence either of small active subunits or of larger aggregates with or without carrier proteins, and may also reflect species differences. Ingeniously, using the expression-

secretion system of a yeast (*Pichia Pastoris* GS115), it has proved feasible to produce recombinant polypeptides of HSA. Various polypeptides thus obtained were added to rat pituitary cultures and only those corresponding to subdomain IIIB (specifically residues 490-585) presented attenuin bioactivity, while the whole molecule and even the whole subdomain IIIB were inactive (Tavoulari et al., 2004). More recently, Karligiotou et al. (2006) used the retrotranscriptase-polymerase chain reaction (RT-PCR) technique and appropriate primers to amplify different transcripts from HSA gene in granulosa cells. Results showed that while all HSA fragments were expressed in the nucleus, only two fragments (the promoter and a C-terminal fragment) were expressed in granulosa cell cytoplasm, indicating a differential expression of the HSA gene, probably leading to attenuin synthesis. Finally, the current attenuin purification strategy involves the use of phage display techniques to produce antibodies against partially-purified human-granulosa-luteal cell-conditioned medium. Three of these antibodies were found to block human attenuin bioactivity on GnRH-induced LH secretion from rat pituitary cell cultures. Subsequently, these antibodies were used to immunopurify attenuin; the antigen-antibody complexes obtained were separated by electrophoresis. Candidate attenuin spots (around 66 kDa and pI 5.5-6.0) were excised for peptide mass mapping. The main molecules identified were HSA precursor and variants. All these data suggest that attenuin may be a post-translationally modified form of serum albumin, or else may be very tightly bound to, and transported by, serum albumin (Sorsa-Leslie et al., 2005).

1.3 Synthesis and secretion pattern of attenuin

In women, the production of attenuin in the ovarian follicle is clearly related to follicular size in both spontaneous and stimulated cycles. FF from follicles smaller than 11 mm (stimulated cycles, Fowler et al., 1994b) or 6-8 mm (spontaneous cycles, Fowler et al., 2001) has been found to contain the greatest amount of attenuin. In women receiving fertility treatment, there is an increase both in the number of follicles and in serum attenuin bioactivity with respect to spontaneous cycles (Byrne et al., 1993). Similarly, small follicles in pig ovaries contain the highest attenuin concentrations, while bioactivity falls sharply in preovulatory follicles (Kita et al., 1994). Thus, medium from granulosa cell cultures showed more attenuin bioactivity when these cells were obtained from aspiration of small follicles (Seo & Danforth, 1994). All these findings suggest that small and growing follicles are the major producers of attenuin. In summary, attenuin levels are highest during the early part of the menstrual cycle (days 1 to 8), falling thereafter as follicular size increases, and disappearing at the appropriate preovulatory time (Fowler et al., 2003). The corpus luteum does not appear to produce attenuin in women, but small developing follicles may produce attenuin during the luteal phase (Messinis et al., 1996; fig. 1 lower panel).

2. Methods of study

2.1 In vivo

In the absence of purified bioactive molecule, for the reasons indicated above, the effect of attenuin on the LH surge in rats is currently studied using both *in vivo* and *in vitro* procedures. The *in vivo* approach consists in administration of exogenous FSH during the diestrous phase in cycling rats as a tool to increase the biological activity of endogenous attenuin (Geiger et al., 1980; Gordon et al., 2008). In addition to attenuin, FSH also stimulates the synthesis and secretion by granulosa cells of a number of steroidal and non-steroidal

factors (mainly estradiol and inhibin) that affect pituitary gonadotropin secretion (Arai et al., 1996; Watanabe et al., 1990). However, this treatment attenuates the magnitude of the proestrous afternoon LH surge despite the presence of high circulating levels of estradiol (Geiger et al., 1980; Gordon et al., 2008, de Koning et al., 1987; see fig. 2A for additional details).

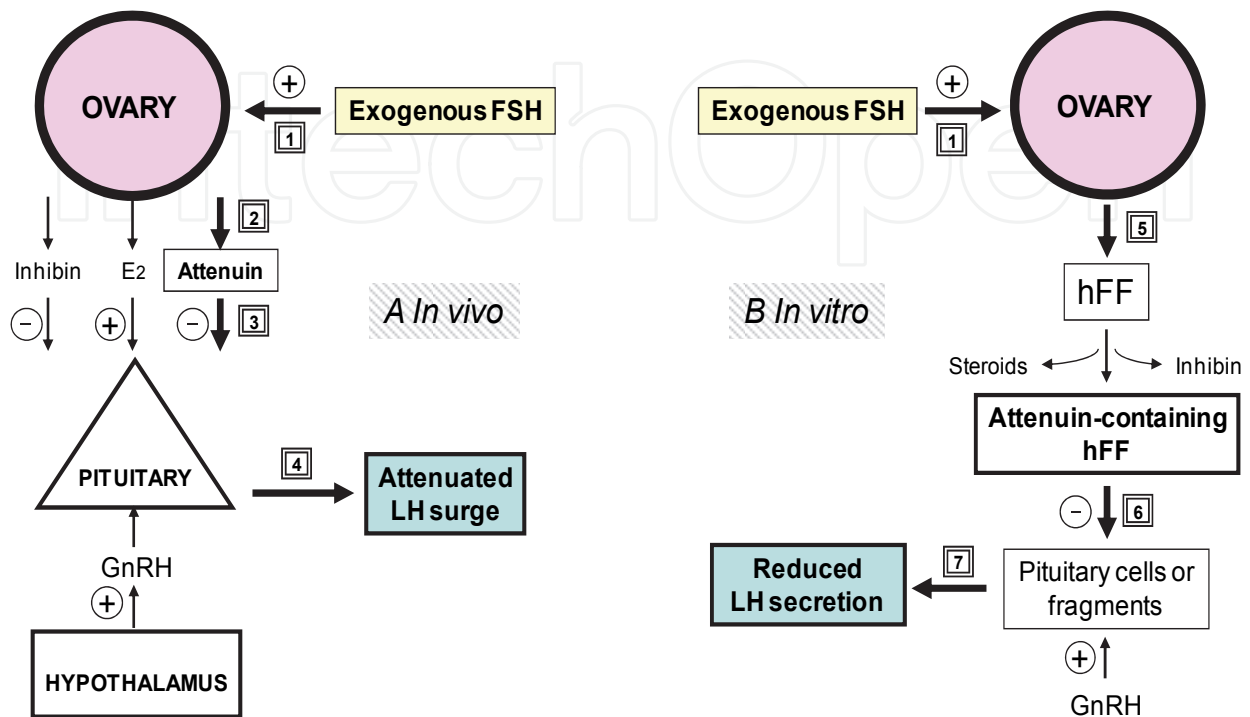


Fig. 2. Schematic representation of current approaches to the study of attenuin effects on luteinizing hormone (LH) secretion. GnRH: gonadotropin releasing hormone; E2: 17 β -estradiol; FSH: follicle-stimulating hormone; hFF: human follicular fluid. A: *in vivo*. [1] Administration of FSH stimulates the synthesis/secretion of ovarian hormones (E2, inhibin and attenuin) [2]. Whereas in proestrus inhibin suppresses FSH secretion (Gordon et al., 2010a, 2010c) and E2 acts on LH secretion in its positive feedback mode [2], attenuin acting on gonadotrope membrane receptor [3] results in a reduction of the magnitude of the LH surge [4]. B: *in vitro*. Administration of FSH stimulates follicular growth and recruitment [1]. Pooled hFF from women undergoing IVF treatments [5] is submitted to steroid charcoal-extraction and inhibin immunoprecipitation (Gordon et al., 2010c). Attenuin-containing hFF is then added to cultured pituitary cells or fragments [6]. This results in a reduction of GnRH-stimulated LH secretion [7].

At the same time, the bioactivity of attenuin can be promoted and/or prolonged by removing endogenous inhibin and consequently increasing endogenous FSH. One option is the neutralization of inhibin's biological activity by injection of an anti-inhibin serum on metestrus that increases FSH serum levels during diestrus and proestrus, induces superovulation and again reduces the magnitude of the proestrous afternoon LH surge (Ishigame et al., 2004).

2.2 In vitro

However, it is difficult to delineate, *in vivo*, the precise bioactivity of attenuin in the rat pituitary using exogenous FSH. Alternatively, attenuin bioactivity can be studied by *in vitro*

procedures, using cultured anterior pituitary cells or pituitary fragments treated with murine FF (Busbridge et al., 1990; Fowler & Spears, 2004), pFF (Danforth & Cheng, 1995) or hFF from women undergoing IVF treatments (Byrne et al., 1995a, 1996; Fowler & Templeton 1996) as a source of exogenous attenuin, following steroid and inhibin depletion (see fig. 2B for additional details).

3. Mechanism of attenuin action

3.1 Background

Despite considerable research and the availability of improved molecular techniques, little is known about how attenuin acts. This section outlines the key findings reported to date. *In vitro* experiments have highlighted the potentiating effects of increased intracellular calcium, stimulation of both intracellular GnRH signalling pathways (calcium- and cAMP-dependent protein kinases, PKC and PKA respectively), as well as progesterone treatment on GnRH-induced LH secretion (Kile & Nett, 1994; Sánchez-Criado et al., 2006). A further clue regarding the mechanism by which attenuin acts was discovered when, in rat pituitary monolayers, GnRH was co-incubated with either estradiol, progesterone, PMA (phorbol 12-myristate 13-acetate, a stimulator of PKC) or calcium ionophore. All treatments potentiated the effects of GnRH on LH synthesis and release, but administration of hFF counteracted the enhancing effects of all these compounds, with one exception: the augmentative effect of progesterone on GnRH-induced LH synthesis (Cowking et al., 1995). Shortly afterwards, the authors found that while progesterone augmented GnRH self-priming in rat pituitary monolayers, this secretion was blocked when hFF and the antiprogestagen RU486 were added to the medium (Byrne et al., 1995b). All these findings suggest that the mechanism of action of attenuin on preovulatory LH secretion might involve the pituitary PR. There is considerable evidence, both *in vivo* and *in vitro*, to suggest that attenuin has a suppressant effect on GnRH self-priming (Byrne et al., 1996; Koppelaar et al., 1992, 1993). This action has been shown to be linked to a blockade of GnRH second messenger pathways rather than to competition between attenuin and GnRH for GnRH receptors (Fowler et al., 1994c; Tijssen et al., 1997). A study by Helder et al. (1997) reported that the unknown attenuin receptor located in the gonadotrope membrane might act through the cAMP pathway. It might thus be hypothesized that the inhibiting effect of attenuin on LH secretion is exerted through this GnRH second pathway. Both hypotheses, i.e. attenuin acting through a GnRH second pathway or through the PR, are feasible, since it has been shown that GnRH can activate the PR in a ligand-independent manner through the PKA and/or PKC pathways (Garrido-Gracia et al., 2006; Turgeon & Waring, 1994).

3.2 The relationship between attenuin and LH release

A number of published studies report a reduction of the preovulatory LH surge *in vivo* in rats treated with FSH during metestrus and diestrus (Culler, 1992; Geiger et al., 1980; Gordon et al., 2008; de Koning et al., 1987; Shuiling et al., 1999). Additionally, this FSH treatment *in vivo* causes an *in vitro* suppression of GnRH-stimulated LH secretion, GnRH self-priming and progesterone-potentiating effect on GnRH-stimulated LH secretion, without affecting basal LH levels (Byrne et al., 1996; Gordon et al., 2008; Koppelaar et al., 1992, 1993). *Pace* Geiger et al. (1980), the possibility that these effects are produced by an increase in estradiol levels can be ruled out, since animals treated with estradiol benzoate display greater LH secretion both *in vivo* and *in vitro*; more important, FSH plus estradiol-

injected ovariectomized rats do not display any suppressant effect on LH secretion (Gordon et al., 2008). All this proves conclusively that FSH treatment stimulates the production of some ovarian factor(s) other than estradiol, that reduces the preovulatory LH surge but not basal LH secretion. Subsequent *in vitro* research has shown that attenuin produces this inhibiting effect on pituitary sensitivity to GnRH in a dose-dependent manner: administration of different doses of FSH (0.1, 1 and 10 I.U.) caused variable inhibition of LH secretion, partially reversed by simultaneous administration of estradiol (Gordon et al., 2009a). Attenuin would thus appear to bind to its gonadotrope membrane receptor and activate a secondary pathway in order to inhibit preovulatory LH secretion (Fowler & Templeton, 1996). The potentiating effects of progesterone, 8-bromo cAMP and PMA on GnRH-stimulated LH secretion are antagonized in rat hemipituitaries incubated with steroid-free bFF (bFF) or with FF from women undergoing a superovulation protocol (Cowking et al., 1995; Tijssen et al., 1997). All this suggests that attenuin may exert a suppressant effect on the GnRH secondary pathway. Subsequent research has shown that neither progesterone, nor GnRH, nor activation of PKA and PKC has any effect on GnRH-stimulated LH secretion in FSH-treated cyclic rats (Gordon et al., 2008; 2009b), indicating that this effect is probably produced downstream of PKA, PKC and progesterone. Given these findings, and the fact that attenuin decreases all PR-dependent parameters of LH secretion, it may be postulated that this action is exerted through a modification of PR expression and/or action (Garrido-Gracia et al., 2007).

3.3 The relationship between attenuin, PR and LH release

With regard to the effect of FSH-treatment on PR gene expression in rats, RT-PCR analysis has revealed a partial decrease in PR mRNA (Gordon et al., 2008). Nevertheless, immunohistochemical examination revealed no difference in the number of PR-positive pituitary cells in FSH injected rats (Gordon et al., 2008, 2009b) or rats in which inhibin was immunoneutralized; the latter group, however, did not display a similar partial decrease in PR mRNA (Gordon et al., 2010a). These differences may be due to different FSH and/or attenuin levels between experimental groups. In most species and tissues, PR is expressed by a single gene but transcribed into separate mRNAs as two distinct molecular forms, PR-A and PR-B. The B (stimulatory) form contains an additional N-terminal sequence and is the main transcription factor, while the A form is modulatory of B in nature (Vegeto et al., 1993; Wen et al., 1994). For this reason, the present authors decided to analyze two inhibiting possibilities: an increase in PR-A and a decrease in PR-B protein levels. While passive immunization against inhibin with an anti-inhibin serum attenuated preovulatory LH secretion in cyclic rats, pituitary protein levels of both PR isoforms were unaffected by the absence of inhibin. This was the first time the two PR isoform protein levels had been demonstrated using the western blot technique in rat pituitaries (Gordon et al., 2010a). Although attenuin could exert its negative action on LH secretion by several mechanisms, the results pointed to a possible involvement of attenuin in post-translational modifications of PR, leading to its inactivation. Phosphorylation at serine residues is the most frequent post-translational processing event in PR modification (Beck et al., 1992; Takimoto & Horwitz, 1993) and is involved in PR-regulated gene transcription (Denner et al., 1990; Faus & Haendler, 2006). To explore this question, two studies were carried out: the first sought to measure PR phosphorylation levels, while the second aimed to determine the effect of phosphatases on pituitary sensitivity to GnRH.

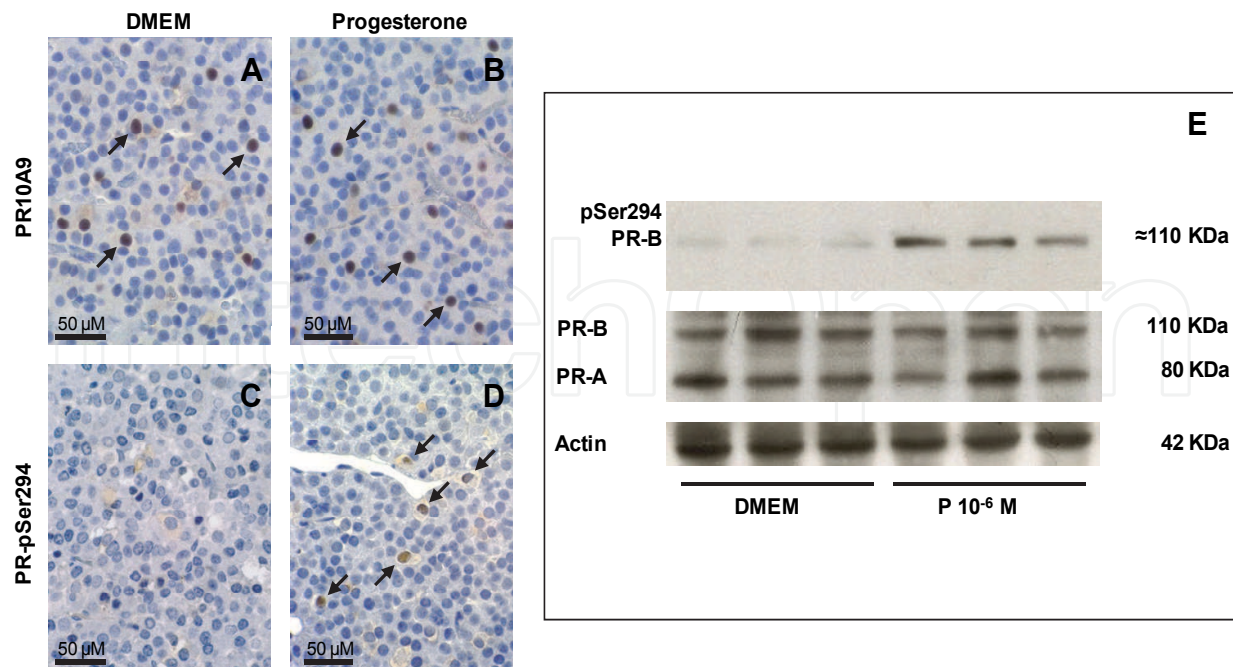


Fig. 3. **Methods of studying progesterone receptor (PR) phosphorylation in rat gonadotropes.** Left panel: Immunoreactive products to PR10A9 antibody in the nuclei of gonadotropes, the only rat pituitary cell expressing PR (Garrido-Gracia et al., 2008). Pituitaries were incubated with medium alone (A, DMEM) or progesterone (B). No differences were found between the number of PR positive gonadotropes. Immunohistochemical expression of phosphorylated PR gonadotropes (C, D) in anterior pituitaries from proestrous rats (Gordon et al., 2009b). Immunoreactive products to pSer294 antibody are observed only in gonadotropes of pituitaries incubated with progesterone (D). The relative expression of pSer294-positive gonadotropes and PR10A9-positive gonadotropes provides an approximation of the PR phosphorylation rate. Right panel: PR A and B isoforms and phosphorylated PR-B content in pituitaries from proestrous rats incubated either with medium alone (DMEM) or with progesterone ($P 10^{-6}$ M). Only PR-B phosphorylated product is seen in pituitaries incubated with progesterone (Gordon et al., 2011).

In the first study, the PR phosphorylation protocol in pituitaries was optimized by adding progesterone to the incubation medium. Subsequently, pituitaries from rats treated with FSH or with anti-inhibin serum were immunostained with an antibody that recognizes the immunogen corresponding to amino acid residues 288–300 from human PR-B, Ser294 being phosphorylated (Clemm et al. 2000). All the experimental groups in which attenuin was increased and preovulatory secretion diminished showed a significant drop in the number of cells expressing pSer294 (Gordon et al., 2009b, 2010a; fig. 3, left panel).

The following study analyzed the *in vitro* effect of calyculin, a potent inhibitor of intracellular phosphatases (Condrescu et al. 1999), on GnRH-stimulated LH secretion and GnRH self-priming in FSH-treated rats. The results showed that this drug was able, albeit partially, to reverse GnRH-stimulated LH secretion in a dose-dependent manner (Gordon et al., 2009b). Altogether, these results suggest that the ovarian-dependent inhibitory effect of FSH injection on the preovulatory LH secretion in the rat may involve an imbalance between the activities of protein kinases and phosphatases, resulting in a dephosphorylation of pituitary PR (see fig. 4 for additional details). In the light of previous

immunohistochemical data (Gordon et al., 2009b, 2010a; fig. 3, left panel), the preliminary results obtained using the western blot technique (Gordon et al., 2011; fig. 3 right panel) supported the hypothesis that attenuin produces an inhibiting effect on LH secretion by dephosphorylation of pituitary PR.

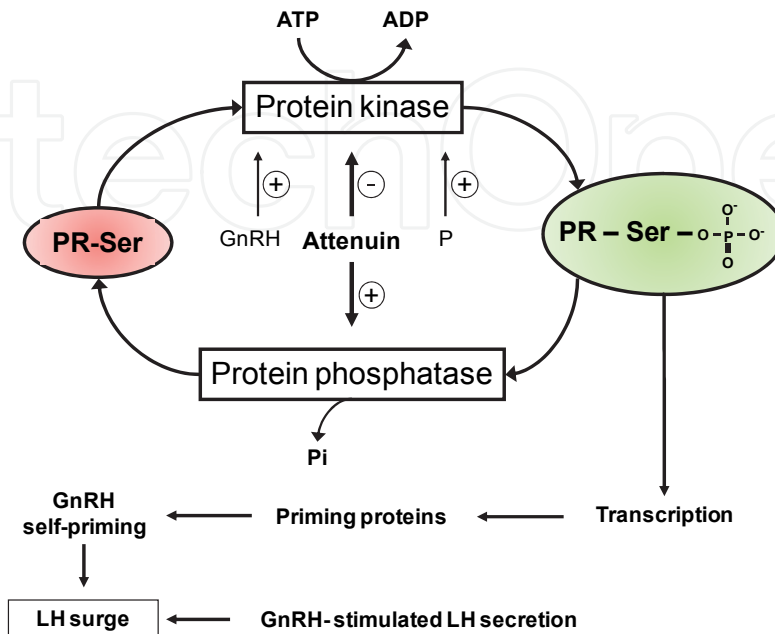


Fig. 4. **Proposed effects of attenuin on the LH surge.** GnRH: gonadotropin releasing hormone; PR-Ser: gonadotrope unphosphorylated progesterone receptor (PR); PR-Ser- PO_4^{2-} : gonadotrope phosphorylated PR. PR can be activated/phosphorylated in a ligand-dependent (progesterone) or independent (GnRH second pathways) manner. Attenuin reduces phosphorylation of Ser (serine) of the immunogen MAPGRS(p)PLATTV located in the N-terminal domain of PR-B. Activated PR-B transcribes “priming proteins” resulting in GnRH self-priming. This unique property of gonadotropes, together with estradiol sensitization of gonadotropes to GnRH, results first in GnRH-stimulated LH secretion and finally in the LH surge.

3.4 The relationship between attenuin and LH synthesis

The preovulatory secretion of LH is the result not only of increased LH synthesis (Tse et al., 1993) but also of increased LH secretion by gonadotropes (Ramey et al., 1987). While the effects of attenuin on LH secretion have been exhaustively studied, it is not known whether attenuin has any effect on LH synthesis. Fowler et al. (1995b), using rat pituitary cultures, studied the effects of steroid-free and inhibin-depleted hFF on GnRH- augmented LH synthesis and secretion in rat pituitary cultures. Results showed a decrease in both parameters, suggesting that this factor reduces pituitary responsiveness to GnRH by suppressing both the *de novo* synthesis and the acute and long term release of LH. However, there is no evidence to show how attenuin might effect this suppression. The present authors found that the addition of ionomycin (a calcium ionophore) to the incubation medium did not result, as expected, in a massive release of LH in pituitaries from FSH-treated rats. Moreover, no differences were found in LH secretion between calyculin plus GnRH and ionomycin, indicating a possible failure in LH synthesis (Gordon et al., 2009b). LH pituitary content and LH β mRNA expression were therefore evaluated; results showed

that FSH treatment decreased pituitary LH content in intact, but not in ovariectomized, rats injected with estradiol benzoate, without affecting LH β mRNA levels. Altogether, these results suggest lower LH synthesis caused by attenuin, potentiating its inhibiting effects on LH secretion (Gordon et al., 2009a).

3.5 The relationship between attenuin, PR and LH synthesis

To determine whether pituitary PR was involved in LH synthesis inhibition by attenuin, cyclic rats were injected with different doses of FSH and progesterone. Results showed that while progesterone by itself had no effect (control groups), saturation of PR with the cognate ligand reversed the inhibitory effect of attenuin on LH protein levels, although not on LH secretion (Gordon et al., 2010b). Apart from these results showing that attenuin probably inhibits LH synthesis at post-transcriptional level, little is known about the mechanism involved in that inhibition – probably by increasing LH degradation (Kitahara et al., 1990) and/or decreasing GnRH-induced LH polypeptide glycosylation (Ramey et al., 1987) – except that, as indicated earlier, the inhibitory pathway in LH synthesis involves the gonadotrope PR. However, the detailed mechanism underlying the PR-mediated attenuin-induced inhibition of LH protein levels requires further detailed investigation. Even so, the available evidence supports the view of PR as a keystone in the neuroendocrine integrator at both hypothalamic and pituitary levels (Levine et al., 2001). In conclusion, it is postulated that, just as inhibin controls FSH synthesis and release (Attardi et al., 1991; Scott & Burger 1981), attenuin reduces proestrous GnRH-dependent LH secretion through a dual mechanism of action: inhibition of both LH synthesis and LH release.

4. The possible physiological role of attenuin

4.1 Humans

Menstrual cyclicity in women is highly dependent on positive and negative ovarian feedback mechanisms. During the follicular phase of the cycle, estradiol plays a key role in the control of both gonadotropins. Together with this steroid, low concentrations of circulating progesterone and inhibin B also contribute to the control of LH and FSH secretion, respectively. During the luteal phase, both estradiol and progesterone regulate secretion of the two gonadotropins, while inhibin A plays a role in FSH secretion. The transition from follicular to luteal phase involves a preovulatory secretion of both LH and FSH, helped by the estradiol positive mode (fig. 1, left panel). However, the change from negative to positive estradiol feedback at some threshold cannot entirely account for the change from pituitary insensitivity to the maelstrom of events leading to the LH surge. Although the question remains unresolved, there is evidence that estradiol and attenuin interact on the pituitary in the context of the positive feedback mechanism. It may be assumed that estradiol sensitizes the pituitary to GnRH, while attenuin antagonizes that sensitizing effect. Based on existing knowledge regarding attenuin action and its secretion pattern, it has been suggested that attenuin activity is greater during the early and midfollicular phases and lower both in the late follicular phase and at midcycle. Therefore, the pituitary LH response to GnRH is low during the early and midfollicular phase and is markedly enhanced during the late follicular phase, triggering the full expression of the preovulatory LH surge. According to this hypothesis, the role of attenuin in humans is to control the amplitude and not the onset of the LH surge (Messinis et al., 2006). It should be highlighted the possible role of attenuin in the polycystic ovary syndrome (PCOS). In PCOS,

the mechanism responsible for abnormal gonadotropin secretion (elevated serum LH) has not been completely elucidated. One possible etiopathology mechanism is an increased of pituitary responsiveness to GnRH, caused by a decreased in attenuin production (Ruiz et al., 1996). This would offer an explanation for the most common endocrine disease that affects ovulation and fertility. At the same time, and once attenuin had been isolated, this peptide could be used as a GnRH antagonist and as a physiological contraceptive agent.

4.2 Rats

Administration of FSH during the diestrous phase prompts a reduction in proestrous LH secretion in the rat. It is worth noting that the effects of attenuin on proestrus can only be observed with this treatment (Culler, 1992; Geiger et al., 1980; Koppenaal et al., 1991). However, it is difficult to determine with any precision the role of attenuin in physiological conditions using this experimental model. Previous findings by the present authors (Gordon et al., 2010c) give grounds for speculation regarding the possible physiological role of attenuin. The incubation protocol for pituitary glands from intact cycling rats on each of the 4 days of the estrous cycle demonstrated the existence of GnRH self-priming in diestrous and proestrous phases. Subsequently, steroid-free inhibin-depleted hFF was added to the medium. Results reported by Fowler et al. (Fowler et al., 1994b, 2001, 2003) suggest a reduction in attenuin synthesis and/or secretion when the ovarian follicle is close to preovulatory size. For this reason, we used hFF from follicles of two sizes: small (<15 mm in diameter) and large (>15 mm in diameter). Surprisingly, results in terms of secretion levels showed that only diestrous GnRH self-priming, but not proestrous self-priming as expected from the effects of injected FSH, was susceptible to the inhibiting action of attenuin contained in hFF. It should be noted that hFF from both large and small follicles had the same effects on all the LH and FSH secretion parameters so far studied. Nevertheless, the possibility that a different inhibitory bioactivity of hFF may be found using hFF from smaller follicles cannot be ruled out (Fowler et al., 2003; Fowler & Spears, 2004). Overall, these facts were interpreted as signifying that, during the normal estrous cycle, ovarian attenuin inhibited pituitary PR-dependent GnRH self-priming in diestrus only. Later on, in proestrus, pituitaries become either desensitized to the inhibitory bioactivity of attenuin and/or sensitized to the stimulatory activity of GnRH (fig. 1, right panel). By contrast, Tijssen et al. (1997) showed that attenuin was able to suppress both diestrous and proestrous GnRH self-priming. These discrepancies may be due to the different origins of FF (human vs. bovine), to the experimental model used (static vs. dynamic incubation) and to the absence or presence of inhibin. Previous results published by this research group (Tébar et al., 1996, 1998) support the hypothesis that the pituitary loses sensitivity to attenuin when follicles reach the preovulatory size, which would account for estradiol suddenly exerting a positive feedback. This intriguing mechanism, by which pituitaries become insensitive to attenuin and/or sensitive to the stimulatory action of GnRH, may provide an extra clue for understanding estrous cycle length regulation. This factor would limit the physiological timing (proestrous afternoon) as well as the magnitude (preovulatory secretion) of pituitary responsiveness to GnRH. This research thus shows how a decrease in endogenous FSH levels during diestrus, with a consequent reduction in attenuin production but no significant effect on estradiol levels, gives rise to a 1- or 2-day advancement of blunted preovulatory LH surges in 4- (Tébar et al., 1996, 1998) and 5-day (Sánchez-Criado et al., 1996) cyclic rats, respectively. This suggests that, in physiological circumstances, submaximal FSH-dependent ovarian attenuin bioactivity prevents the premature LH surge in diestrus by

antagonizing the secretory effect of GnRH (Koppelaar et al., 1991; Tébar et al., 1998) and/or the sensitizing effect of estradiol on the pituitary (Schuiling et al., 1999). On the whole, all these findings, together with the existence of pituitary PR on diestrus, would suggest that PR-dependent GnRH self-priming in the diestrous phase in the 4-day cyclic rat is blocked by attenuin bioactivity.

5. Conclusions

Our work over the last few years suggests that attenuin is a FSH-dependent ovarian factor different from inhibin, which decreases preovulatory LH secretion in rats by activating gonadotrope membrane receptors, resulting in PR dephosphorylation. The reduction in PR activity is associated with a decrease in both LH secretion and LH synthesis.

The bioactivity of attenuin appears to play a major role in synchronizing physiological pituitary and ovarian events, so that preovulatory LH secretion is limited to proestrus, when the ovarian follicle and the oocyte are in optimal conditions to respond to the LH surge and to be fertilized, respectively.

However, all these findings must be viewed as speculative until experiments can be repeated using purified attenuin.

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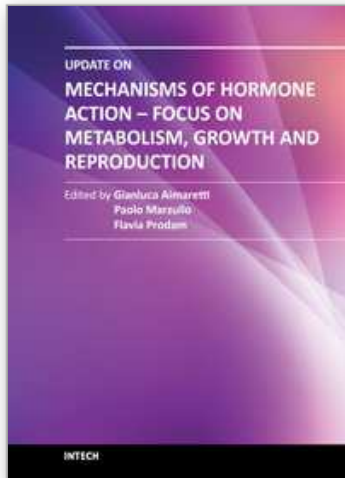
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The purpose of the present volume is to focus on more recent aspects of the complex regulation of hormonal action, in particular in 3 different hot fields: metabolism, growth and reproduction. Modern approaches to the physiology and pathology of endocrine glands are based on cellular and molecular investigation of genes, peptide, hormones, protein cascade at different levels. In all of the chapters in the book all, or at least some, of these aspects are described in order to increase the endocrine knowledge.

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