

Age-Related Analysis of Inhibin A, Inhibin B, and Activin A Relative to the Intercycle Monotropic Follicle-Stimulating Hormone Rise in Normal Ovulatory Women

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Previous studies have reported that the monotropic rise in FSH in older women is associated with decreased inhibin B and/or A levels and increased levels of activin A. Whereas most investigators have found decreased follicular-phase inhibin B, the roles of inhibin A and activin A as modulators of the FSH rise are unclear. The objectives of this study were to determine whether deficiencies in circulating levels of inhibin A, inhibin B, and/or activin A exist during the intercycle interval in ovulatory older (age, 40–45 yr; n = 16), compared with younger women (age, 20–25 yr; n = 13). Blood samples were obtained daily throughout one menstrual cycle and the fol-

licular phase of the subsequent cycle and were analyzed for LH, FSH, estradiol, inhibin A and B, and activin A. Despite significant FSH elevation, no deficiencies in inhibin A, activin A, or estradiol were detected in older subjects. In fact, inhibin A was significantly higher in older participants during the intercycle phase ($P = 0.01$), whereas inhibin B was significantly lower. Thus, the monotropic rise in FSH does not appear to result from changes in inhibin A or activin A, supporting the concept that inhibin B plays a critical role in mediating the FSH rise in older women. (*J Clin Endocrinol Metab* 89: 2977–2981, 2004)

HUMAN OVARIAN SENESCENCE is a continuous process that begins many years before the event of menopause, which marks the cessation of normal ovarian function. To understand the etiology of human ovarian aging, it is prudent for us to study ovarian aging at its earliest stages. The most consistent endocrine change observed in ovulatory women as they enter into the period of diminished ovarian function is the monotropic rise in FSH (1–4). To date, the underlying factors responsible for this monotropic rise in FSH have not been completely elucidated. Recent studies have suggested that decreased follicular and/or luteal production of the inhibin protein hormones (5–11) and/or an increase in activin levels (9, 10) may accompany the monotropic FSH rise. Although an age-related decrease in early follicular phase inhibin B has been consistently reported, whether inhibin A and activin are critical modulators of the early follicular phase monotropic FSH rise remains unclear.

During the normal menstrual cycle, FSH begins to rise in the late luteal or early follicular phase, reaches a peak in the early follicular phase, and subsequently falls throughout the remainder of the follicular phase in response to negative feedback from ovarian steroids and/or inhibin (12, 13). The monotropic FSH elevation observed in older women is present throughout the menstrual cycle; however, it is most pronounced in the early follicular phase (3, 4). In older women with regular menses, the early follicular phase FSH

peak occurs earlier in the cycle and reaches a greater magnitude (4).

To fully understand the relative roles of inhibin and activin in reproductive aging, it is important to carefully examine changes in their serum profiles during the luteal follicular transition or intercycle period (the interval encompassing the late luteal phase of one cycle and the early follicular phase of the subsequent cycle). Thus, the objectives of the current study were to determine whether reproductive aging in normal ovulatory women is associated with changes in circulating levels of inhibin A, inhibin B, and/or activin A during this critical intercycle period.

Subjects and Methods

Subjects

As part of a series of clinical studies to examine the normal reproductive aging process, we recruited healthy, ovulatory women aged 40–45 yr (n = 16) and 20–25 yr (n = 13) for participation in this study. All participants were required to have regular menstrual cycles, normal body mass index (18–24 kg/m²), absence of medical or reproductive disorders (including any history of infertility), and midluteal serum levels of prolactin less than 20 pg/ml, progesterone more than 10 nmol/liter, and testosterone less than 3 nmol/liter in a prestudy cycle. Women who engaged in greater than 5 h/wk of aerobic exercise were excluded. For the duration of the study, all participants were either sexually abstinent or used nonhormonal methods of contraception. Written consent was obtained, and monetary compensation was provided to all volunteers. The protocol was reviewed and approved by the University of Washington Human Subjects Review Committee.

Protocol and procedures

To completely capture the intercycle FSH peak, daily blood samples were collected during one complete menstrual cycle and continued

Abbreviation: CV, Coefficient(s) of variation.

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throughout the subsequent follicular phase in all volunteers. All serum samples were analyzed in duplicate in the same assay to minimize effects of interassay variability. In addition, participants underwent daily transvaginal ultrasound monitoring of dominant follicle development beginning in the midfollicular phase and continuing until follicle collapse was observed. Ultrasound examinations were performed by one of the investigators using an Ultramark 400C (ATL Ultrasound, Bothell, WA) with a 6.5-MHz vaginal transducer. Follicle sizes were estimated by calculating the mean of the two greatest perpendicular diameters in a two-dimensional plane.

Serum FSH levels were determined using a solid-phase two-site monoclonal ELISA (DELFLIA, Wallac Inc., Gaithersburg, MD). The intra- and interassay coefficients of variation (CV) were 1.9 and 8.9%, respectively.

Samples were analyzed for LH by an immunoradiometric method (MAIA clone; Serono Laboratories, Geneva, Switzerland). The intra- and interassay CV were 3.9 and 14.6%, respectively.

The RIA for serum estradiol was performed using reagents supplied by ICN Biomedicals, Inc. (Costa Mesa, CA). The intra- and interassay CV were 9 and 18%, respectively.

Serum P was determined by solid-phase RIA using reagents supplied by Diagnostic Systems Laboratories, Inc. (Webster, TX). The antibody cross-reactivity is less than 5% with all other steroids. The intra- and interassay CV were 11 and 13%, respectively.

A solid-phase sandwich ELISA (Serotec, Oxford, UK) was used to measure inhibin B, based on the use of plates coated with a monoclonal antibody specific for the inhibin β -B subunit with a second monoclonal antibody specific for the inhibin α -subunit for detection. The sensitivity was 15.6 pg/ml, and inhibin A had a 0.5% cross-reaction in the inhibin B ELISA. The assay was controlled in triplicate using samples with mean concentrations of 155.3, 316.3, and 919.3 pg/ml, with interassay CV of 11.6, 7.6, and 9.7%, respectively.

Inhibin A was analyzed by a solid-phase sandwich ELISA (Diagnostic Systems Laboratories). This assay uses a monoclonal first antibody specific for the β -A subunit of inhibin, with a horseradish peroxidase-labeled monoclonal second antibody specific for the α -subunit for detection. The assay standard provided by the manufacturer was calibrated using the World Health Organization's First International Standard for Inhibin (recombinant human inhibin; lot 91/624), and results are reported as international units per milliliter of this reference material. Sensitivity of the assay was 0.1 IU/ml. The assay was controlled in duplicate using aliquots of specimens containing 2.00 or 9.56 IU/ml, with interassay CV of 8.7 and 3.3%, respectively.

Activin A was measured by a solid-phase sandwich ELISA (Diagnostic Systems Laboratories) using monoclonal antibody specific for the β -A subunit followed by a biotinylated monoclonal antibody and streptavidin-alkaline phosphatase for detection. Samples were pretreated with sodium dodecyl sulfate to avoid interference of bound follistatin. The detection limit is 10 pg/ml, and recovery is 102 + 3% for bovine follicular fluid and 96 + 5% for human serum. Cross-reactivity is less than 0.5% for inhibin A, B, and activin B and less than 0.1% for pro- α -C and follistatin. The assay was performed in duplicate with control samples of 0.15, 1.25, and 7.5 pg/ml with intra/interassay CV of 4.5 and 14, 3.4 and 12, and 7.5 and 11%, respectively.

Statistical analysis

Results are expressed as means \pm SE. Two-way ANOVA with repeated measures was used to test for differences between groups over the menstrual cycle. Single-point measurements were compared using a Student's *t* test. Statistical significance was defined as $P < 0.05$ in all cases.

Results

The characteristics of the study participants are shown in Table 1. The first of the two menstrual cycles for each subject was selected arbitrarily for d 3 and follicular phase comparisons. As shown previously, older women had higher levels of FSH on cycle d 3, lower levels of inhibin B, and shorter follicular phase length compared with the younger control group. The FSH elevation was most pronounced in the in-

TABLE 1. Study subject characteristics (means \pm SEM)

	Younger (n = 13)	Older (n = 16)	<i>P</i>
Age (yr)	23.5 \pm 0.4	42.6 \pm 0.4	<0.01
Day 3 FSH (IU/liter)	5.7 \pm 0.4	10.4 \pm 1.5	<0.01
Day 3 estradiol (pmol/liter)	159 \pm 11	278 \pm 88	NS
Day 3 inhibin B (pg/ml)	110 \pm 10	69.4 \pm 12	0.02
Follicular phase length (d)	15.4 \pm 1.0	12.7 \pm 0.5	0.02
Luteal phase length (d)	13.1 \pm 0.4	13.6 \pm 0.4	NS
FSH intercycle peak (IU/liter)	6.9 \pm 0.5	13.1 \pm 1.9	<0.01
Day of intercycle FSH peak	4.1 \pm 0.5	2.9 \pm 0.4	0.06

NS, Not significant.

tercycle phase (intercycle peak: older, 13.1 + 1.9 IU/liter *vs.* younger, 6.9 + 0.5 IU/liter; $P < 0.01$) between the two cycles studied. There were no significant differences in cycle d 3 estradiol levels or luteal phase length (first cycle). All participants developed a dominant follicle with ultrasound demonstration of ovulation noted after the LH surge.

Figure 1 depicts levels of FSH, LH, estradiol, progesterone, inhibin A, inhibin B, and activin A across the menstrual cycle when normalized to the LH surge. Again, as expected, FSH was elevated throughout the initial cycle and the subsequent follicular phase in the older group. There were no significant differences in LH, activin A, or in luteal-phase progesterone levels. There was a trend toward higher activin A in the older group, consistent with previous reports, although this trend did not reach statistical significance and was diminished when data were normalized to the early follicular phase FSH peak (Fig. 2). Estradiol was significantly elevated in the older women, most apparently in the follicular phase during dominant follicle development. There was also a trend toward higher levels of inhibin A (not significant), whereas inhibin B was significantly lower in the older participants.

Figure 2 depicts levels of FSH, estradiol, inhibin A, inhibin B, and activin A, examined in the intercycle phase (the 5 d immediately surrounding and including the early follicular phase FSH peak). When data were normalized to the early follicular phase FSH peak, the older women had significantly lower levels of inhibin B and similar levels of estradiol and activin A. In contrast, inhibin A levels were significantly greater in the older subjects than in the younger controls.

Discussion

The monotropic FSH rise in older reproductive-age women has been well documented and recognized for many years by numerous investigators (1–4). When it became clear that this rise precedes any significant decline in ovarian steroid secretion (3, 4), attention turned to inhibin as the most likely candidate ovarian hormone to account for FSH elevation. Previous studies using older assays that measured total immunoreactive inhibin, as well as its free subunits and precursors, confirmed that inhibin levels fall with advancing age and rising FSH, ultimately becoming undetectable after menopause (14–16). After development of assays specific for dimeric inhibin A and B (17, 18), it became evident that the two hormones exhibited very different patterns across the menstrual cycle. Inhibin A, the predominant inhibin product of the dominant follicle and corpus luteum, has a pattern in the follicular phase that is similar to estradiol, rising and

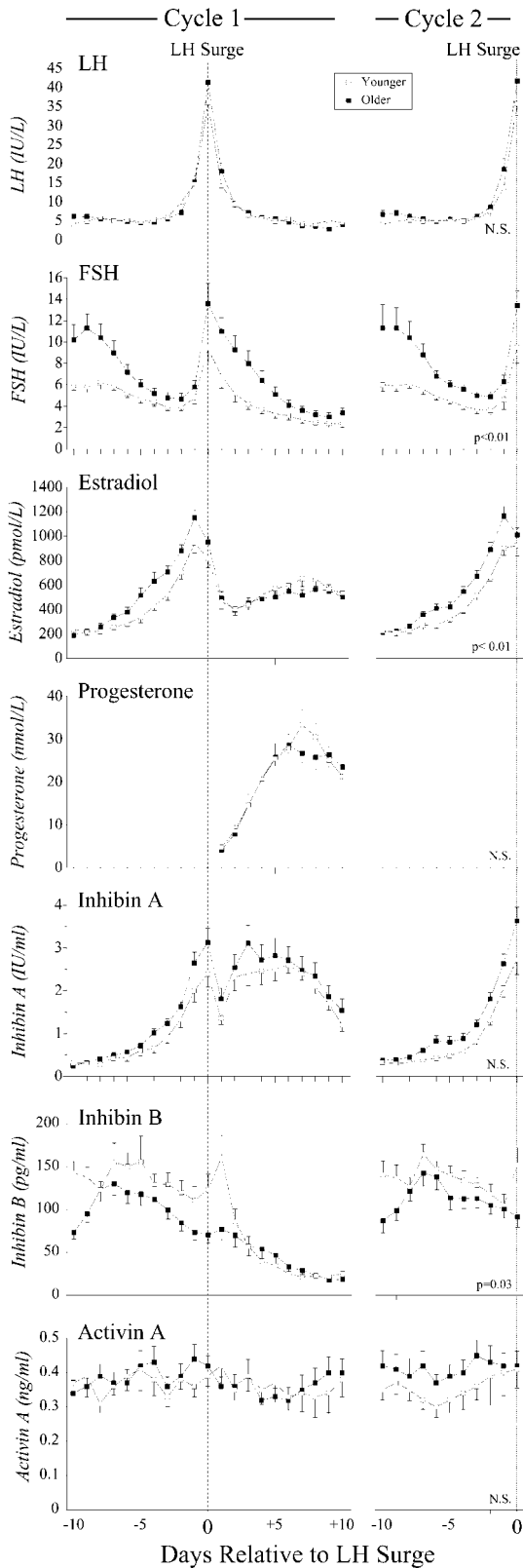


FIG. 1. Peripheral levels of LH, FSH, estradiol, progesterone, inhibin A, inhibin B, and activin A normalized to the LH surge in the two consecutive cycles in older subjects (40–45 yr, n = 16) and younger controls (20–25 yr, n = 13). The values are shown as mean ± SEM. Significant differences are noted above each graph.

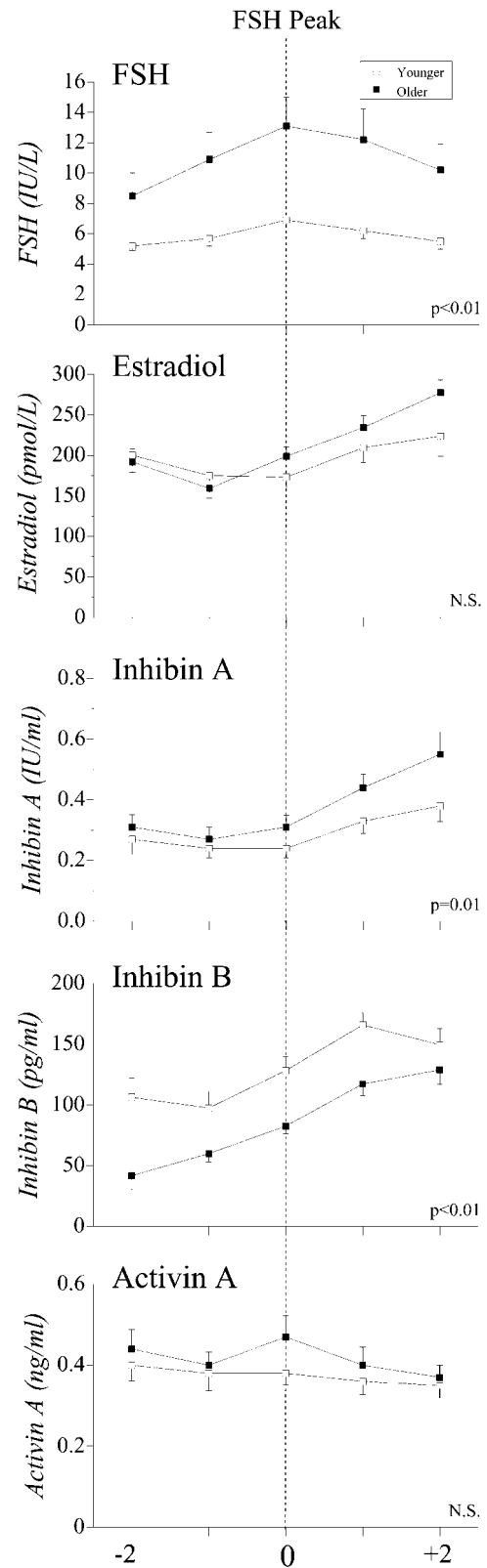


FIG. 2. Peripheral levels of FSH, estradiol, inhibin A, inhibin B, and activin A normalized to the intercycle FSH peak in older subjects (40–45 yr, n = 16) and younger controls (20–25 yr, n = 13). The values are shown as mean ± SEM. Significant differences are noted above each graph.

reaching a peak with maturation of the dominant follicle (18). It falls transiently with ovulation and then rises and remains elevated throughout the luteal phase. Inhibin B is the predominant inhibin in the small antral follicles, rising in the early to midfollicular phase, then falling throughout the late follicular phase and remaining low for the duration of the luteal phase (18).

Using assays specific for dimeric inhibin, several investigators have reported that inhibin B is low in the follicular phase of older women exhibiting a monotropic FSH elevation (6–11). Inhibin B levels inversely correlate with FSH and positively correlate with the number of small (<5 mm) antral follicles present by transvaginal ultrasound (19). It is likely that this decrease in inhibin B concentration is the result of fewer primordial and early antral follicles remaining in the ovaries of older women (20, 21). Previous studies of inhibin A secretion in reproductive aging have produced conflicting results, with some investigators reporting low inhibin A secretion (5, 6, 8, 10) and others reporting normal levels in older, ovulatory women (7, 9, 11). In the present study, there was no evidence that inhibin A is deficient in early stages of reproductive aging after the onset of the monotropic FSH rise. The fact that overall inhibin A secretion appears to be higher in the older women suggests that it is inhibin B and not inhibin A that is responsible for the monotropic FSH rise. This is consistent with previous evidence that inhibin A is not a major determinant of the early follicular phase FSH rise in normal reproductive-age women (9, 22). Others have reported that inhibin A was normal in older women without FSH elevation but was decreased in older participants who demonstrated a monotropic FSH rise (8). Welt *et al.* (11) found no differences in luteal phase levels of inhibin A between older (age, 35–46 yr) and younger (age, <35) ovulatory women; however, inhibin A levels declined with age in all three women studied longitudinally. Thus, it appears that inhibin B but not A declines in the earliest stages of reproductive aging, in contrast to the decrease in inhibin A levels that has been previously reported (5, 6, 8, 10, 11). Taken together, available data on inhibin A suggest that a decrease in this hormone may represent a later development during reproductive aging.

In contrast to previous reports by other investigators (9, 10), the current study did not detect a significant increase in circulating activin A levels. Possible explanations include relatively small sample sizes and differences in the characteristics of the study populations. Activin A has been shown to increase with age in both men and women and does not correlate with FSH levels in either gender (23, 24). Furthermore, mRNA for activin subunits is expressed in a number of extragonadal tissues (25), is present predominantly in a bound (and therefore inactive) form in the circulation (26), and does not vary across the menstrual cycle despite marked variability in FSH levels. Although our current understanding of activin physiology is limited by the lack of available assays for other activin forms (*e.g.* activin B, activin AB), to date there is little evidence to support an endocrine role for activin in FSH regulation.

Similar to the elevated estradiol (11, 27, 28) and normal progesterone levels (3, 4) observed in older women demonstrating a monotropic FSH rise, normal or increased inhibin

A may be a result of FSH elevation. In previous studies of 40- to 45-yr-old ovulatory women, we found normal follicular fluid concentrations of steroids and inhibin (28–30). Together with normal peripheral levels of progesterone and normal to increased levels of estradiol and inhibin A, these findings suggest that, under the influence of FSH elevation, the dominant follicle is fully functional in terms of its secretory capacity. Thus, FSH elevation may represent a compensatory mechanism in response to declining inhibin B secretion from a diminishing follicle pool, sufficient in early stages to maintain normal dominant follicle development and ovulation. By recruiting only women who reported very regular menstrual cycles, we selected women who were in a relatively early stage of reproductive aging (*i.e.* equivalent to stage 4 or 3 according to the Stages of Reproductive Aging Workshop) (30). Cross-sectional and longitudinal studies reported by others (5, 11) suggest that inhibin A levels eventually decline as women approach the menopausal transition, which represents a more advanced stage of reproductive aging.

Previous studies of inhibin secretion have been limited by sampling during only one menstrual cycle. Due to the dynamic interactions of hormones across the menstrual cycle, comparisons between groups require normalizing data to a specific point in the cycle (traditionally, the midcycle LH surge). Relative to the menstrual period, older reproductive-age women have an earlier onset of the monotropic FSH rise (4) and shorter follicular phase length (1, 4, 23, 31) such that normalization to the LH surge does not allow for comparison of hormones in the early follicular phase. The onset of menses is an end-organ response to falling ovarian steroids and, as such, is a crude and inaccurate indicator of the beginning of the follicular phase (13). Therefore, to study the relationship between inhibin and FSH, it was important to examine the entire intercycle phase so that the intercycle FSH peak could be identified and the associated hormonal patterns compared relative to this important physiological time point.

In summary, this study more precisely characterizes the role of the inhibin and activin glycoprotein hormones in early reproductive aging. The finding of an isolated inhibin B deficiency in the face of normal estradiol, activin A, and inhibin A supports the notion that inhibin B may be the primary mediator of the monotropic rise in FSH. Thus, a decline in inhibin B may be a sensitive marker of the earliest stages of ovarian senescence.

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