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Alterations in plasma and ovarian immunoreactive inhibin during reproductive aging in the female rat

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Previously, we showed that ovarian inhibin α- and β_A-subunit mRNAs are elevated in middle-aged and old persistent-estrous (PE) female rats. To determine whether higher inhibin subunit mRNA expressions result in increased circulating inhibins during reproductive aging, plasma immunoreactive inhibin α (ir-inh α) and gonadotropins were measured in young, middle-aged and PE rats. Plasma LH profiles were distinctly divergent in the middle-aged rats with some showing LH surges indistinguishable from young rats and others showing significantly attenuated LH surges. Plasma ir-inh α in middle-aged rats with LH surges were similar to those of young rats. However, animals with attenuated LH surges had higher peak ir-inh α levels than young and middle-aged animals with LH surges. Immunohistochemistry revealed increased levels of ovarian inhibin α staining in those animals with attenuated LH surges. Overall, the highest plasma and ovarian inhibin α were found in PE rats which lack LH surges. However, significant decreases of plasma and ovarian inhibin α were seen after reinstatement of estrous cyclicity with progesterone implant treatment. Thus, increases in both plasma and ovarian inhibin α appear to be closely associated with attenuation or loss of the preovulatory gonadotropin surge that occurs during aging.

Keywords: inhibin; aging; LH; ovary; immunohistochemistry

Introduction

The reproductive cycle of the female rat is critically dependent on regulated endocrine interaction between ovarian and pituitary hormones that accompany follicular growth, atresia, ovulation and corpora lutea formation. One of the key hormones involved in the coordination of these processes appears to be the ovarian glycoprotein inhibin. Inhibin is produced mainly by the granulosa cells of developing follicles and has been shown to be involved in the preferential inhibition of pituitary FSH secretion (DePaolo *et al.*, 1979; Rivier *et al.*, 1986; Hasegawa *et al.*, 1987; Vale *et al.*, 1988; Ying, 1988; Haisenleder *et al.*, 1990). In addition, several recent studies have indicated that inhibin may also play a role in the endocrine modulation of pituitary LH secretion (Lumpkin *et al.*, 1984; Culler & Negro-Vilar, 1989; Rivier & Vale, 1991; Culler, 1992a,b).

The changes in inhibin subunit gene expression and secretion are closely regulated by pituitary gonadotrophins and ovarian steroids as well as by various paracrine/autocrine factors (Bicsak *et al.*, 1986; Suzuki *et al.*, 1987; Zhiwen *et al.*, 1987). Characterization of inhibin subunit mRNA levels and immunoreactive inhibin levels during the estrous cycle substantiates a functional feedback relationship between inhibin production and gonadrotropin secretion in the cyclic adult female rat (DePaolo *et al.*, 1979; Hasegawa *et al.*, 1987; Meunier *et al.*, 1988, 1989; Haisenleder *et al.*, 1990). Indeed, experimentally or pathologically induced changes in the levels of inhibin have been shown to result in significant alterations of pituitary gonadotropin secretion and ovarian follicular development (Rivier *et al.*, 1989; Culler, 1992a,b).

In our previous study we have demonstrated that higher levels of inhibin α- and β_A-subunit mRNAs are expressed in the ovaries of cyclic middle aged (9–11 month old) female rats compared to cyclic young (3–4 month old) female rats during the periovulatory period (Jih *et al.*, 1993a). Substantial elevations of inhibin subunit gene expressions were also observed in the ovaries of acyclic old (12–14 month old) persistent-estrous (PE) female rats (Jih *et al.*, 1993b). Interestingly, in both middle-aged and PE rats the changes in inhibin subunit gene expressions were associated with alterations in the patterns of the preovulatory LH and FSH release. Given that only inhibin gene expression was examined in the previous study, it is unclear if age-associated increases in the levels of inhibin mRNA also result in higher levels of inhibin secretion. In the present study, we measured and correlated the levels of plasma immunoreactive inhibin α (ir-inh α) and plasma gonadotropins around the periovulatory period in individual rats of different reproductive ages. The cell specific distribution of inhibin α-subunit production was also examined in the ovaries by immunohistochemistry.

Results

Patterns of plasma gonadotropins and immunoreactive inhibin α (ir-inh α) in young and middle-aged female rats

Plasma LH and FSH in the young animals began to surge at proestrous (P)1500 h and P1700 h, respectively (Figure 1). The secondary FSH surge occurred around E0300 h. Plasma ir-inh α levels in young animals increased during proestrous afternoon and was the highest around the time of peak LH levels at P1900 h. This increase in plasma ir-inh α was significant ($P<0.05$), and was followed by a significant decline in ir-inh α levels by P2100 h ($P<0.01$). Plasma ir-inh α continued to remain low throughout estrous morning (Figure 1c). The levels of plasma ir-inh α measured for the young female rats were similar to values previously reported for female mice using this same inhibin radioimmunoassay system (Pal *et al.*, 1991).

The preovulatory LH surge in the middle-aged female rats showed distinct variations in magnitude. Some middle-aged animals had attenuated LH surges while others displayed LH surges indistinguishable from those of young animals (Figure 1a). In middle-aged animals with clear LH surges, a significant ($P<0.05$) increase in plasma LH above baseline levels was first detected at P1700 h. LH levels in these animals at P1700 and P1900 h were not significantly different from those of young animals. Middle-aged animals were subsequently divided into two groups based on their LH surge profile (surge *vs* non-surge) for the analyses of plasma FSH and ir-inh α values.

The middle-aged female rats which had clear LH surges also had clear biphasic FSH surges (Figure 1b). A large

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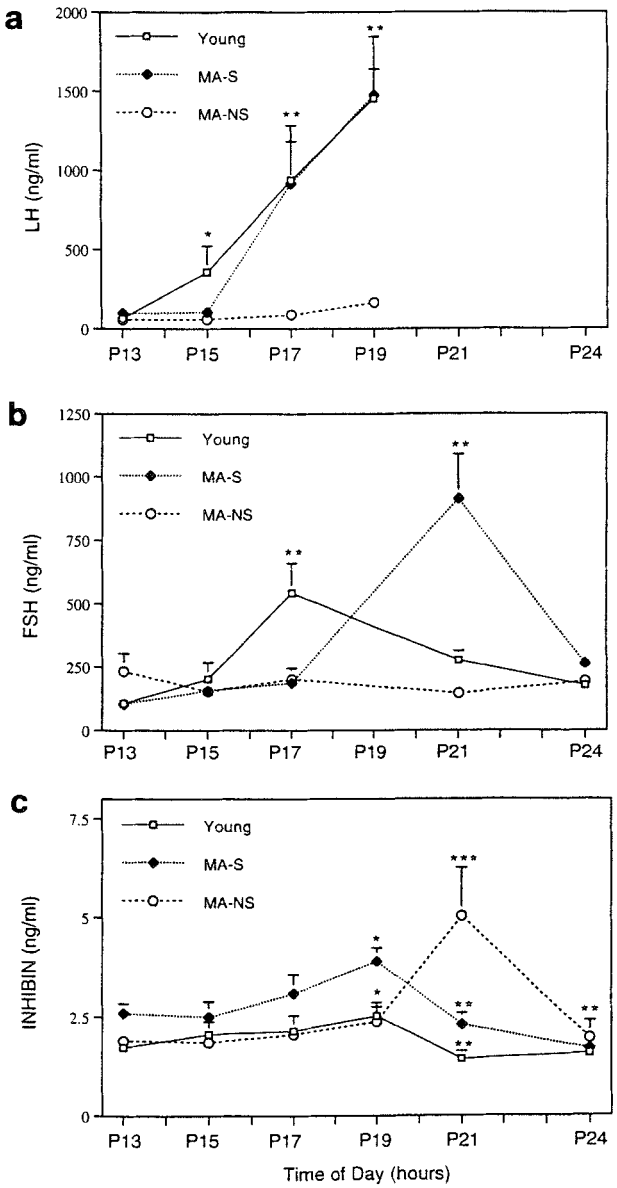

Figure 1 Patterns of mean plasma LH, FSH and ir-inh α during the periovulatory period in young, middle-aged surge and middle-aged non-surge female rats. Plasma LH (**a**), FSH (**b**) and ir-inh α (**c**) levels were measured by RIA in young ($n = 6$), middle-aged surge ($n = 5$) and middle-aged non-surge ($n = 5$) female rats. Plasma FSH and ir-inh α measurements in middle-aged female rats were analysed based on their LH surge profile – surge (MA-s) *vs* non-surge (MA-NS). Values are the mean ± SEM. P, poestrus; E, estrus. $*P<0.05$ (compared to baseline at P1300 h for LH and ir-inh α in young and MA-S animals). $**P<0.01$ (compared to baseline at P1300 h for LH and FSH, and compared to peak levels for ir-inh α). $***P<0.01$ (compared to young and MA-S animals)

increase in plasma FSH was detected at around P2100 h ($P<0.01$) and was followed by a second significant increase ($P<0.01$) in plasma FSH around E0300 h. ANOVA with repeated measures revealed significant age-related increases in the overall levels of plasma ir-inh α in the middle-aged surge rats compared to young rats ($P<0.01$). However, the patterns of plasma ir-inh α were similar to those of young female rats with levels of inhibin gradually rising to peak levels ($P<0.01$) during proestrous afternoon and significantly decreasing ($P<0.01$) by P2100 h (Figure 1c).

The group of middle-aged female rats with significantly blunted LH surges also failed to show significant elevations in FSH above baseline levels (Figure 1b). There were no significant differences in basal LH or FSH levels between young, middle-aged surge and middle-age non-surge animals. However, the levels of ir-inh α were significantly higher ($P<0.01$) at P2100 h in the middle-aged animals with attenuated LH surges compared to both young and middle-aged surge rats (Figure 1c). An eventual disease in ir-inh α levels occurred in these middle-aged non-surge animals at P2400 h ($P<0.01$).

Patterns of plasma gonadotropins and immunoreactive inhibin α (ir-inh α) in PE and progesterone implant (P-implant) treated female rats

On the first proestrus after p-implant removal all animals had clear LH and FSH surges (Figure 2a and b). The magnitude of LH surges in P-implant treated PE rats did not differ significantly from those of either young or middle-aged surge rats. However, the timing of the LH surges appeared slightly advanced (significant increase above baseline at P1300 *vs* P1500 h in young female rats and *vs* P1700 h in middle-aged rats) in the P-implant treated PE animals. The FSH surge in P-implant treated animals also appeared to be advanced (peak at P1300 and P1500 h), and there did not appear to be a clear secondary FSH surge on early estrous morning (Figure 2b). Control PE animals did not display LH surges and had low plasma LH levels (271.1 ± 24.4 ng/ml) similar to basal levels found in cyclic young, middle-aged and P-implant treated animals. Untreated PE animals also did not have FSH surges. However, constant basal FSH levels were higher in PE animals (521.3 ± 23.3 ng/ml) than in both young and middle-aged female rats ($P<0.05$).

Untreated control PE female rats had the highest circulating levels of ir-inh α of all groups studied (4.64 ± 0.56 ng/ml). In contrast, at all time points examined, ir-inh α levels in the P-implant treated animals were significantly lower ($P<0.01$) compared to those of control PE female rats. The overall patterns and levels of plasma ir-inh α resembled those of young cyclic female rats with increases in ir-inh α levels on proestrus afternoon and significantly decreased ir-inh α levels on late proestrus and early estrous morning (Figure 2c).

Ovarian inhibin α-subunit expression in young female rats

The patterns of ovarian inhibin α-subunit production in young cyclic female rats were similar to those previously reported (Meunier *et al.*, 1988). On P1700 h moderately high levels of inhibin α staining were observed in the granulosa cells of tertiary follicles as well as in the theca interna and ovarian stroma (Figure 3A). In the stroma, inhibin α was predominantly localized to discrete islands of secondary interstitial cells. Positive immunoreactivity was also noted in follicle-like cell masses which are the hypertrophied theca of atretic follicles (Figure 3B). After the LH surge the staining in the granulosa cells of large preovulatory follicles was notably decreased by P2400 h (Figure 3B). However, inhibin α immunoreactivity in the interstitial cells did not decrease substantially until next morning at E0900 h. At that time only low levels of inhibin α staining were found in granulosa cells of developing secondary follicles (Figure 3C). At all time points examined inhibin α immunoreactivity was undetectable in small developing follicles or in corpora lutea.

Patterns of ovarian inhibin α-subunit peptide expression in middle-aged surge and non-surge female rats

Ovaries from individual middle-aged female rats were separated into two groups based on the LH surge profiles. The patterns of ovarian inhibin α-subunit immunoreactivity in animals with clear LH surges were similar to those of young rats throughout the time points examined. Specifically, levels of inhibin α staining in granulosa cells of developing

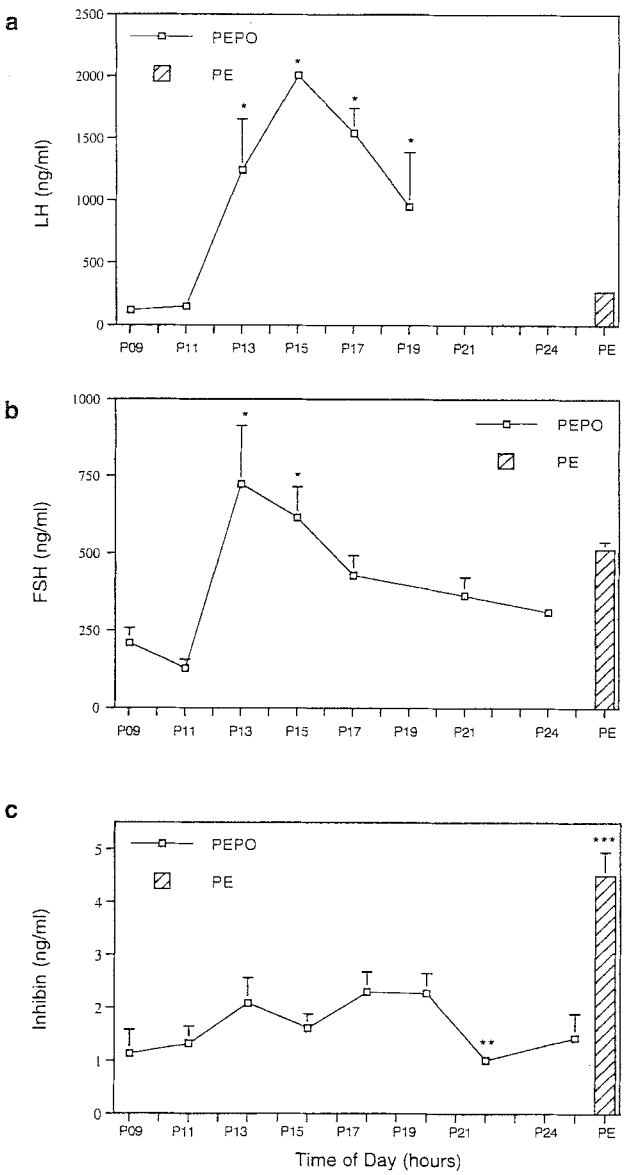

Figure 2 Plasma LH, FSH, and ir-inh α levels in the old PE (**PE**) rats and in PE rats after progesterone implant treatment (**PEPO**). PE rats received progesterone implants for 6 weeks. After removal of implants and return of estrous cyclicity, serial blood samples were taken for plasma LH (**a**), FSH (**b**), and ir-inh α (**c**) measurements. Blood samples were also taken at 1300 h from five untreated PE controls. Each value represents the mean ± SEM from 4–5 animals. $*P<0.01$ (compared to baseline for LH and FSH, and for PE compared to PEPO). $**P<0.05$ (compared to peak levels at P1900 h for ir-inh α). $***P<0.01$ (compared to PEPO plasma ir-inh α levels at all time points).

follicles increased during proestrus (Figure 3D) and significantly decreased by P2400 h after completion of the preovulatory LH surge (Figure 3E). A moderate to high level of staining was also observed in the interstitial gland cells of these ovaries. As in young rat ovaries, inhibin α immunoreactivity in the interstitial glands decreased notably only after ovulation as evidenced by the overall lower levels of immunofluorescence in ovaries from E0900 h (Figure 3F).

In middle-aged rats which did not display significant LH surges, ovarian immunoreactive inhibin α increased during proestrous afternoon (Figure 3G), but failed to show any clear decrease at P2400 h (Figure 3H). Instead, high levels of inhibin α immunostaining were still apparent in the granulosa cells of large preovulatory follicles at P2400 h. On estrous morning, higher numbers of large preovulatory-like follicles were found in these ovaries than in the ovaries of either young or middle-aged surge animals. These large follicles continued to display high levels of inhibin α-subunit immunostaining (Figure 3I). In addition, moderately high levels of immunostaining were still found in the ovarian interstitial cells of these ovaries on estrous morning.

Patterns of ovarian inhibin α-subunit peptide expression in PE and P-implant treated PE female rats

The ovaries from PE female rats displayed very high levels of immunostaining for the inhibin α-subunit. Strong immunostaining was observed in the granuloa cells and theca interna of large growing and cystic follicles, and substantial amounts of staining was seen in the ovarian stroma (Figure 3J). Very high levels of inhibin α immunoreactivity was almost uniformly present in the ovary with the exception of large degenerating cysts devoid of granulosa cells (data not shown).

In contrast, there was a definite and clear decrease in the overall levels of immunoreactive inhibin α-subunit staining in the ovaries from P-implant treated female rats. Although the low number of apparently healthy large follicles found on proestrous afternoon did display strong inhibin α immunoreactivity, a dramatic decrease in the levels of stromal inhibin α staining was evident (Figure 3K). In addition, compared to PE ovaries there were higher numbers of cysts in the P-implant treated ovaries. No immunostaining for the inhibin α-subunit was seen in the numerous large cystic structures which do not possess a granulosa cell layer (Figure 3K). In addition, the newly formed corpora lutea were also negative for inhibin α-immunostaining. The controls which were incubated with either primary antibody preabsorbed with synthetic inhibin α peptide or PBS exhibited no immunostaining (Figure 3L). Summaries of ovarian inhibin α immunoreactivity in young, middle-aged surge and non-surge, and PE animals are presented in Table 1.

Discussion

Although middle-aged animals are comprised of a heterogeneous population with some animals showing clear LH surges and some showing significantly attenuated LH surges (Wise, 1982; Nass *et al.*, 1983; Lu *et al.*, 1985 and this present study), the overall plasma ir-inh α levels in both middle-aged groups were significantly higher than in young animals. The patterns of plasma ir-inh α in the middle-aged animals with clear LH surges were similar to those of young animals while the patterns of plasma ir-inh α in the middle-aged animals with attenuated LH surges differed significantly from those of both young and middle-aged surge animals. Middle-aged animals with LH surges of similar magnitude to those of young animals still exhibited a slight delay in the onset of the LH surge (P1700 h *vs* P1500 h in young animals), and also had higher levels of ir-inh α on proestrous and estrous morning than young animals. Therefore, small but significant increases in ovarian ir-inh α secretion may occur even in the absence of any significant changes in the preovulatory LH secretion. Chronically high levels of ir-inh α in anovulatory PE female rats that do not display the LH surge indicate that elevations in ir-inh α secretion are associated with impairments of LH secretion during aging. Similar elevations in plasma ir-inh α levels in PE rats were also noted in a previous study (Matt *et al.*, 1993).

Overall, greater levels of ir-inh α inhibin were also found in the ovaries of the middle-aged non-surge and PE female rats compared to the ovaries of young and middle-aged surge female rats. The highest inhibin α immunoreactivity were found in the large cystic follicles of the PE ovary. Due to lack of ovulation, high levels of immunoreactive inhibin α are likely to be continually present and secreted from the PE

ovaries consistent with the present finding of higher plasma ir-inh α in these animals. However, treatment of PE rats with P-implants which reinstated apparently normal estrous cycles and spontaneous LH and FSH surges similar to those of young animals resulted in significant decreases in the levels of both plasma and ovarian immunoreactive inhibin α. In addition, there was a return to a cyclic pattern of immunoreactive inhibin α secretion reflective of the cyclic changes in gonadotropin secretions. Thus, the high levels of immunoreactive inhibin α expression appear to be specifically correlated to the absence of ovulatory cycles rather than to the chronologic age of the animal. These findings corroborate results from our earlier study which showed higher levels of inhibin α- and β_A-subunit gene expressions in the ovaries of middle-aged and PE female rats (Jih *et al.*, 1993a,b), and also indicate that increased inhibin gene expression is closely associated with higher release of inhibin α-subunit peptides.

The present study indicates that an adequate LH and/or

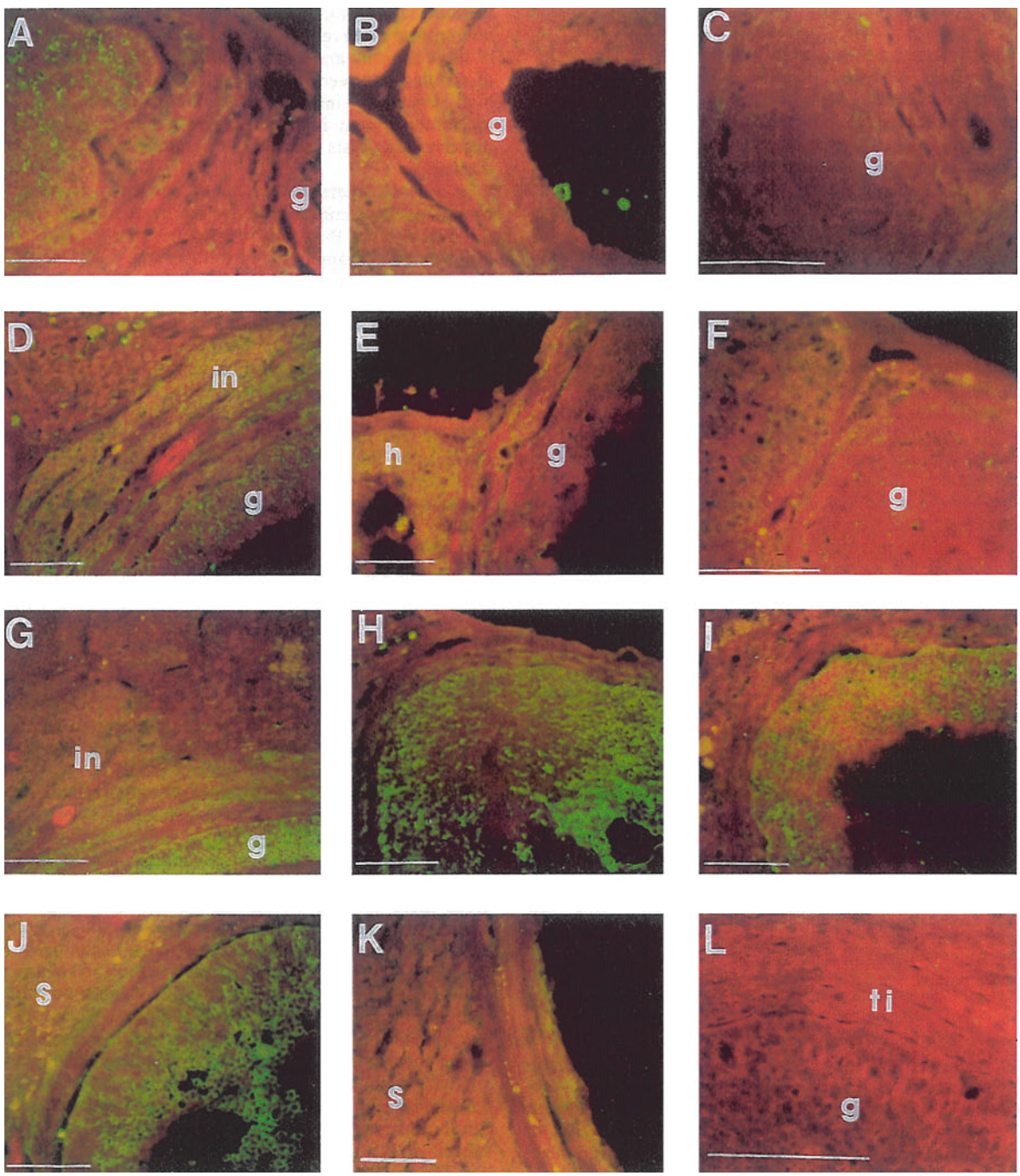

Figure 3 The patterns of immunoreactive inhibin α-subunit expression in the ovaries of young, middle-aged, and old PE female rats. Ovaries were collected from young female rats (**A–C**), middle-aged rats with LH surges (**D–F**), middle-aged rats with attenuated LH surges (**G–I**) at 1700 h (**A, D, G**) and 2400 h (**B, E, H**) on proestrus and at 0900 h on estrus (**C, F, I**). Ovaries were also collected from old PE rats (**J**) and PE rats after P-implant treatment (**K**). Expression of inhibin α-subunit was identified by FITC-conjugated secondary antibody. The sections were counterstained with Evans Blue which distinguishes the negatively staining areas as red *vs* positive FITC staining which fluoresces green. Control sections were incubated with inhibin α antibody preabsorbed with synthetic inhibin peptide (**L**). The bars on the photographs represent 150 μm in length. **g**, granulosa cells; **h**, hypertrophied theca; **in**, interstitial gland cells; **s**, ovarian stroma; **ti**, theca interna

Table 1 Distribution of inhibin α-subunit immunohistochemical staining in young, middle-aged and old persistent-estrous (PE) rats during proestrus (P) and estrus (E)

	Large follicles	*Theca interna*	*Stroma/ISG*
Young			
P1300 h	+ +	+	+
P1700 h	+ + +	+ +	+ +
P2400 h	–	+ +	+ +
E0900 h	–	–	+
Middle-aged surge			
P1300 h	+ +	+	+
P1700 h	+ + +	+ +	+ +
P2400 h	–	+ +	+ +
E0900 h	–	+	+ +
Middle-aged non-surge			
P1300 h	+ +	+	+
P1700 h	+ + +	+ +	+ + +
P2400 h	+ + +	+ +	+ + +
E0900 h	+ +	+	+ +
Old PE			
1300 h	+ + +/–[a]	+ +	+ +
PE-progesterone implant			
P1300 h	+ +/–	+	+
E0900 h	–	+	+

Ovarian sections were analysed under fluorescent microscopy. The relative levels of staining intensities observed in each group are indicated by + or –. Scores were assigned as follows: + + +, intense staining; + +, strong staining; +, low staining; and –, no staining above background. [a]Large cystic follicles expressed high levels of inhibin immunoreactivity while cysts devoid of granulosa cells were negative for inhibin α-subunit peptide expression

FSH surge(s) is requisite for the timely decrease in plasma ir-inh α levels seen at proestrous 2100 h. The increased immunoreactive inhibin α levels found in the old PE rat ovaries and plasma were positively correlated with elevated plasma FSH levels, suggesting that tonic FSH stimulation in the absence of ovulation can chronically enhance ovarian inhibin α production. The reason(s) for the concurrent increases in both ir-inh α and pituitary FSH secretion in aging animals is not clear. The elevated plasma FSH levels without a corresponding decrease in immunoreactive inhibin in the old PE rat ovaries suggest that the negative feedback mechanism of inhibin on pituitary FSH release may not be normal. On the other hand, since only measurements of immunoreactive inhibin α were made, it is possible that the PE ovaries secrete predominantly large quantities of the inhibin α-subunit monomers. Interestingly, recent assays aimed at detecting dimeric inhibin found only very low levels of inhibin in human sera (Groome *et al.*, 1974; Lambert-Messerlian *et al.*, 1994). As yet, there has been no comprehensive study addressing changes in dimeric inhibin levels during the estrous cycle of the female rat (Baly *et al.*, 1993; Woodruff *et al.*, 1993). It is most likely that large portions of the ir-inh α detected in circulation by radioimmunoassays consist of variously processed forms of the inhibin α monomer as well as the dimeric inhibin. However, a potential physiological role of α-inhibin as an autocrine or paracrine FSH modulator has recently been proposed (Schneyer *et al.*, 1991, 1994). Inhibin α-subunit peptides have been found to inhibit FSH binding to both natural and recombinant rat FSH receptors. In addition, purified recombinant α-inhibin proteins were partial FSH antagonists in an *in vitro* bioassay. Potentially, the increased inhibin α-subunit peptides produced in the aging rat ovaries may alter and/or impair normal follicular development by competing for FSH receptor binding.

Inhibin is a sensitive indicator of the changes in the patterns of ovarian follicular development as well as gonadotropin secretion (Woodruff *et al.*, 1987, 1989, Meunier *et al.*, 1988; Rivier *et al.*, 1989). Thus, alterations in the patterns and/or levels of inhibin subunit gene expression and production observed during reproductive aging in the female rat could be merely reflective of changes in both ovarian and pituitary function. However, the association of higher inhibin levels with an attenuated or absent LH surge in the middle-aged and PE female rats may have a functional role as an endogenous gonadotropin surge-inhibiting or attenuating factor (GnSIF/AF) (Culler, 1992a). Studies have shown that high levels of inhibin immunoactivity under ovarian hyperstimulation conditions, such as those induced by exogenous PMSG or FSH as well as direct inhibin administration, can block the preovulatory LH and FSH surges (Lumpkin *et al.*, 1984; Culler, 1992a,b). The inhibition of the LH surge was effectively prevented when carried out in the presence of inhibin antiserum (Culler, 1992a). The suppressive effect of inhibin is apparently due to its ability to repress the self-priming effect of gonadotropin releasing hormone (GnRH) at the level of the anterior pituitary (Wang *et al.*, 1988, 1989). Injection of animals with porcine follicular fluid or recombinant inhibin led not only to decreases in FSH secretion but decreases in basal and GnRH stimulated LH secretion as well (Lumpkin *et al.*, 1984; Culler, 1992b). Although it is still unclear what specific physiologic function(s) inhibin may have during reproductive aging in the female rat, the results of the present study indicate a possible relationship between the changes in inhibin production and LH surge secretion during reproductive aging. Determination of the effects of inhibin on LH secretion by pituitary gonadotropes should provide direct evidence to such a notion.

Materials and methods

Animals

Young (3–4 months) virgin and middle-aged (8–9 months) retired breeder Long-Evans female rats were purchased from Charles River Laboratories (Portage, Michigan) and housed five per cage under standard vivarium conditions. Young animals were used between 3–4 months of age while middle-aged animals were used between 9–11 months of age in the following studies. In the animal room, temperature (24–26°C) and lighting schedules (lights-on 0500–1900 h daily) were controlled throughout the study. Daily vaginal smears were taken from these animals to determine their estrous cycle patterns. Young and middle-aged females that exhibited at least two consecutive 4-day estrous cycles were considered regularly cyclic and used for the following experiments. Old (12–14 months) acyclic female rats which had displayed greater than fifteen consecutive days of cornified vaginal cytology were classified as PE in this study.

Blood sampling and tissue collection

Groups of young and middle-aged female rats were implanted with jugular vein cannulas under light ether anaesthesia on the late afternoon of diestrus-2. Next day on proestrous (P) morning, serial blood samples (0.2 ml) were obtained at designated times (P1300, P1500, P1700, P1900, P2100 and P2400 h) for the measurement of plasma inhibin levels. Plasma LH measurements were made from 0.1 ml blood samples taken at P1300, P1500, P1700 and P1900 h. Previous studies (Wide, 1982; Nass *et al.*, 1983; Jih *et al.*, 1993a) have shown that the LH surges in both young and middle-aged Long-Evans female rats peak around proestrus 1700 h and begin to plateau around 1900 h on proestrous afternoon. Thereafter, LH levels inevitably being to decline after 1900 h. Thus, sampling times for LH measurements were limited to only between P1300 h and P1900 h to insure that surges could be adequately detected, and concomitantly, to minimize blood loss due to sampling. Lastly, a volume of 0.2 ml blood was collected for plasma FSH measurements at P1300, P1500, P1700, P2100, P2400, E0300 and E0900 h. Blood samples from each female were collected with heparinized syringes and immediately centrifuged. Separated

plasma was stored at −20°C for radioimmunoassay. The remaining blood cells were resuspended in heparinized saline and reinfused back into each individual animal.

For tissue collection, groups of female rats ($n = 6$, young and $n = 10$, middle-aged) were killed by decapitation at P1300, P1700, P2400 and E0900 h. Ovaries dissected from each rat were rinsed in ice-cold 0.1 M phosphate buffered saline (PBS) and then fixed by immersion in 4% paraformaldehyde in 0.1 M PBS for 24 h at 4°C followed by cryoprotection in 20% sucrose-PBS. Tissues were embedded in Tissue-Tek medium (Miles Scientific, Naperville, IL) and frozen sections were cut at 6 μm, mounted on Superfrost/Plus microscopic slides (Fisher Scientific, Pittsburgh, PA) and stored at −70°C until processed for immunohistochemistry.

Progesterone implant treatment

Established 12–14 months old PE female rats ($n = 9$) were implanted subcutaneously with a 4 mm long silastic progesterone implant (P-implant) containing 100% crystalline progesterone (Sigma, MO) for a period of 6 weeks. Earlier studies have determined that the effectiveness of these implants diminishes after a 2 week period. Therefore, new implants were placed in the animals at 2 week intervals to maintain maximally effective levels of circulating progesterone throughout the 6 weeks of treatment (Jih *et al.*, 1993b). An additional group of animals ($n = 5$) were left untreated and served as PE controls. P-implant treated animals were implanted with a jugular vein cannula at the time of implant removal for serial blood sampling (same time points as for young and middle-aged rats). From our previous study, it has been shown that after P-implant removal animals initially display 1 to 2 days of leukocytic smear before the first proestrus. Animals were smeared daily to document their return to cyclicity. On proestrous morning, serial blood samples were taken beginning from proestrus 1300 h to estrus 0900 h at designated time intervals (same as for young and middle-aged animals) for the measurement of plasma inhibin, LH and FSH. One group ($n = 4$) of animals were killed at proestrus 1300 h for collection of ovarian tissues. Blood sampling was continued in the remaining animals ($n = 5$) through estrous morning and ovaries were collected at estrus 0900 h. The untreated PE controls were killed at a single time point (1300 h) and trunk blood was collected from these animals also for the measurement of serum LH, FSH and inhibin. The ovaries were removed from each animal and processed as described above for frozen sections.

Hormone radioimmunoassays (RIAs)

LH and FSH were measured by double-antibody RIAs. The rat LH and FSH RIA reagents were provided by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) and the National Hormone and Pituitary Program (Baltimore, MD). The LH and FSH values are expressed in terms of the reference standards, NIDDK rat LH-RP-1 and rat FSH-RP-1, respectively. The intra- and interassay coefficients of variation for LH were 7.9% and 10.3%, respectively, and those for FSH were 7.4% and 11.5% respectively. The sensitivities of the LH and FSH assays were 53 and 88 ng/ml, respectively.

Levels of immunoreactive inhibin α were measured with a commercial porcine double antibody inhibin RIA kit according to directions provided by the manufacturer (Peninsula Laboratories, Belmont, CA). The antibody and standards used for the inhibin assay are based on synthetic peptides of the N-terminal portion (α 1–32) of the porcine α-subunit of inhibin. This antibody has been shown to detect intact inhibin and also cross reacts with free inhibin α-subunits. This antibody, however, does not cross react with substance P, LH-RH, somatostatin-28, or seminal plasma inhibin-like peptide. Since the potential interference of activin, follistatin or α_2 macroglobulin in this inhibin assay is not known, ovariectomized rat sera were included in the inhibin RIA. No detectable immunoreactive inhibin level was found in the ovariectomized rat sera. All collected plasma samples used for the determination of ir-inh α were measured in one complete assay. Previous studies have shown that the immunoreactive inhibin measured using this type of RIA system closely reflect measurements performed using inhibin bioassays and can be used to characterize changes in the levels and/or patterns of inhibin secretion under various physiologic conditions (DePaolo *et al.*, 1979; Schanbacher, 1988; Vaughan *et al.*, 1988; Rivier & Vale, 1989; Haisenleder *et al.*, 1990; Pal *et al.*, 1991). Inhibin concentrations are expressed in terms of the concentration (ng/ml) of the synthetic α-inhibin peptide. Intraassay variation was 2.3% and the sensitivity of the assay at 95% binding was 63 pg/ml.

Immunohistochemistry

Antiserum against the inhibin α-subunit peptide used for the immunohistochemistry was generously provided by Dr Wylie Vale at The Salk Institute (La Jolla, CA). The antibody was raised in rabbits against a synthetic N-terminal fragment (pIa^{1-26}-Gly^{27}-Tyr^{28}-OH) of the porcine inhibin α-subunit (Meunier *et al.*, 1988). The antiserum was diluted to a final concentration of 4 μg/ml in 0.1 M phosphate buffered saline (PBS) containing 3% normal goat serum. The specificity of inhibin α staining was assessed by substituting preabsorbed inhibin α antiserum (incubated for 24 h with 10 μg/ml of a synthetic fragment of the porcine inhibin α-subunit [Peninsula Laboratories, Belmonth CA]) for the inhibin α antiserum. An additional control included the use of 0.1 M PBS in place of the primary antiserum. No specific staining was observed with either of these controls.

Ovarian sections from young, middle-aged, PE and PE progesterone implant treated female rats were all processed simultaneously. The slides were warmed up to room temperature and rinsed in 0.1 M PBS for 5 min at room temperature. Tissue sections were treated with 0.25% triton X-100 in 0.1 M PBS for 30 min to increase antibody penetration. Nonspecific binding sites were blocked by incubating with 30% normal goat serum for 2 h at room temperature. After a brief wash in 0.1 M PBS, sections were incubated with one of the following: (1) inhibin α antiserum, (2) antiserum preabsorbed with synthetic peptide or (3) PBS buffer alone at 4°C for 48 h. Unbound antibodies were removed with three 15 min washes in 0.1 M PBS. For the visualization of the antigen-antibody complex, slides were incubated for 2 h at 37°C with FITC-conjugated goat anti-rabbit immunoglobulin G secondary antiserum (Vector Labs, Burlingame, CA) diluted 1:200 in 0.1 M PBS. After three 15 min washes in 0.1 M PBS, slides were counterstained with 0.01 mg/ml Evans Blue for 1 min. The sections were coverslipped using 80% glycerol in 0.1 M PBS and viewed using an Olympus BH-2 microscope equipped with a high pressure mercury lamp. Photomicrographs were taken with an Olympus C-35AD-2 camera.

Statistics

Statistical analyses of plasma LH, FSH and ir-inh α levels were carried out by ANOVA with repeated measures followed by *post-hoc t* statistical analyses (Tukey's test for least significant difference) for comparison of means within and between groups. The level of significance was set at $P < 0.05$. All values are expressed as the mean ± SEM.

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