Effects of Aging on Luteinizing Hormone Secretion, Ovulation, and Ovarian Tissue-Type Plasminogen Activator Expression

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This study examined the effects of aging on LH surge magnitude, ovulation, and ovarian expression of tissue-type plasminogen activator (tPA), a protease implicated in follicular rupture. While mean LH levels and ovulation rates were similar in middle-aged cyclic and young groups, there was a significant correlation between peak LH levels and ovulation rates in individual rats, such that females with lower LH surges ovulated fewer ova. In a separate experiment, proestrous LH levels were characterized in young and middle-aged rats, followed by *in situ* hybridization analysis of ovarian tPA mRNA. In young proestrous rats, tPA expression was observed in thecal-interstitial cells and oocytes, but not granulosa cells, prior to the LH surge. After the LH surge, there was a marked increase in tPA mRNA levels in granulosa cells of preovulatory, but not smaller follicles, peaking at 0200 hr estrus. By 0500 hr estrus, ovarian tPA expression declined, and ovulation had occurred. In contrast, LH-induced follicular tPA mRNA levels were dramatically lower in middle-aged rats with attenuated LH surges, and persisting preovulatory follicles were common in ovaries of these females on estrus morning. These findings suggest that age-related declines in ovulatory function result in part from altered induction of ovarian tPA expression, likely due to decreased proestrous LH secretion. [E.B.M. 2001, Vol 226:127–132]

Key words: aging; ovulation; luteinizing hormone; plasminogen activator; rat; ovary

Reproductive aging in women and in rodent species is characterized by a decreased pool of ovarian follicles (1–3), altered patterns of gonadotropin and steroid secretion (4–8), and an increased incidence of irregular and anovulatory reproductive cycles (9, 10). The hallmark of reproductive aging in the regularly cyclic female rat is a decline in the magnitude of the preovulatory LH surge on proestrus (4–7). There is considerable heterogeneity among aging rats, such that not all middle-aged females at a given age exhibit attenuated LH surges (5). Interestingly, those that do show decreased LH surge magnitudes soon lose regular ovulatory function (5), suggesting a prognostic value and functional role of altered neuroendocrine regulation of LH release in the eventual onset of irregular ovulatory cycles. However, the immediate impact of attenuated preovulatory gonadotropin surges on ovulation and on the pathways that mediate follicular rupture are not known.

The breakdown of the follicle wall is believed to depend in large part upon the induction of ovarian proteolytic activity by the preovulatory surges of gonadotropins (11, 12). Plasminogen activators (PAs) are serine proteases that cleave the zymogen plasminogen into the active fibrinolytic enzyme, plasmin (13). The PA system is involved in many physiological processes related to tissue remodeling and cell migration (13–15). Although the regulation of plasminogen activation is complex and involves both plasminogen activators and inhibitors, the expression of tissue-type plasminogen activator (tPA) is thought to be an important rate-limiting step regulating ovarian plasmin production and follicle rupture (11, 12, 16–18). Following activation by tPA, plasmin decreases the tensile strength of the preovulatory follicle wall, suggesting a direct effect of plasmin on follicle rupture (11). tPA is expressed in ovarian granulosa cells (19), and tPA activity and mRNA levels are increased in preovulatory follicles (but not smaller follicles) prior to ovulation induced by LH, hCG, and FSH, and by GnRH in hypophysectomized rats (12, 20, 21). The observation that serine protease inhibitors and antibodies to tPA inhibit ovulation (22) suggest further a significant role of tPA in follicle rupture. In addition, PAs and plasmin increase ovarian collagenase activation (23), providing a second mechanism

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through which follicle rupture may be mediated by PAs. Whether or not there are age-related changes in the induction of ovarian tPA expression associated with impaired ovulatory function is not known.

In order to assess potential functional impacts of attenuated LH surges in middle-aged female rats, we have examined the relationship between proestrous LH surge magnitudes and ovulation rates in individual young and middle-aged females displaying regular ovulatory cycles. In addition, we assessed the relationship between attenuated preovulatory LH surges and the pattern of ovarian tPA expression during the periovulatory period. Findings from these studies indicate that decreased LH surges contribute to lower ovulation rates in middle-aged rats, associated with impaired induction of ovarian tPA expression.

Materials and Methods

Animals Young (90- to 100-days-old) virgin and middle-aged (8- to 9-months-old) retired breeder Long Evans female rats (Charles River Laboratories, Portage, MI) were housed five per cage in standard vivarium facilities with controlled room temperature (24–25°C) and lighting schedule (lights on from 0500 hr to 1900 hr daily). Food and drinking water were available *ad libitum*. Daily vaginal smears were obtained to monitor the estrous cycle patterns of these animals. Only those females that showed at least three consecutive 4-day-long cycles were considered to be regularly cyclic, and only young and middle-aged regularly cyclic females were studied. The use of animals in this study was approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

Experimental Procedures. *Characterization of proestrous LH surge.* Young and middle-aged regularly cyclic rats received intrajugular catheters under light ether anesthesia at 1700–1800 hr on diestrous day 2 (5). Animals displaying nucleated vaginal cytology the next day were considered to be in proestrus, and serial blood samples were obtained every 90 min between 1400 hr and 2130 hr that day to characterize the magnitude of the preovulatory LH surge. Blood samples (0.2 ml each) were collected through the catheters into heparinized syringes, and plasma was immediately separated from red blood cells by centrifugation and stored at −20°C until LH radioimmunoassay.

Determination of ovulation rate. Ovulation rates (numbers of ova shed/rat) in sampled rats were determined by examining the oviductal contents at 0900 hr on the morning of estrus, as previously described (24). Female rats were euthanized, and the oviducts were removed and flushed with physiological saline solution to count the numbers of ova shed.

In situ hybridization analysis. The ovarian distribution and level of tPA expression was determined by *in situ* hybridization analysis with a riboprobe corresponding to a 400-bp portion of the rat tPA cDNA (14, 15, 25). The cDNA template was linearized with the restriction enzyme *Sac*I, followed by riboprobe production using T7 RNA polymerase and an *in vitro* transcription system (Promega, Madison, WI). Ovaries were immediately fixed in a 4% paraformaldehyde solution overnight at 4°C, followed by incubation for 16 hr in a 20% sucrose solution. Ovaries were then cryopreserved at −70°C prior to sectioning. Serial sections were cut at 10 μm thickness and mounted on glass slides. Hybridization was performed under standard conditions (50% formamide, 50°C) with the ^{35}S-labeled rat antisense tPA cRNA probe overnight. Slides were then washed under stringent conditions, treated with RNase A to decrease nonspecific hybridization, and washed again. Slides were coated with Kodak NTB-2 emulsion, dried, and allowed to expose for 2 weeks before development. Developed slides were stained with hematoxylin and eosin prior to analyses by light and dark field microscopy. The absence of significant nonspecific hybridization was confirmed by hybridizing tissue sections pretreated with RNase A (data not shown). Intensities of hybridization signals over granulosa cells were determined by a computerized image analysis program (Scion Image, Scion Corporation, Frederick, MD), and expressed as the density of silver grains per area of granulosa cells.

Hormone Assay and Data Analyses. Plasma concentrations of rat LH were measured by double antibody RIA as previously described (5), using reagents from the National Hormone and Pituitary Program, NIDDK, NIH. Intra- and interassay coefficients of variation for the rat LH assay are 5% and 11%, respectively. All samples for a given experiment were performed within the same assay. Differences in hormone levels, ovulation rates, and tPA hybridization intensities between groups were performed using two-way analysis of variance, followed by Student–Newman–Keuls test to determine differences among groups. Correlations between peak proestrous LH values and ovulation rates in individual animals were analyzed by linear regression analysis using Pearson's Product Moment test. A confidence level of $P < 0.05$ was considered statistically significant.

Results

Proestrous LH Surge Magnitude and Ovulation Rates in Young and Middle-Aged Female Rats. Previous reports indicate a decline in LH surge magnitude and a gradual decrease in ovulation rate in some, but not all middle-aged cyclic rats, as compared to young females (4–6, 26). In the present study, middle-aged rats tended to display lower mean LH values on proestrous afternoon than young rats (Fig. 1), although this difference was not statistically significant ($P = 0.131$; $n = 8$–12 rats/group). The mean numbers of ovulating ova observed in the oviducts on estrous morning also did not differ between these same young (11.0 ± 1.1 ova shed/rat) and middle-aged (10.7 ± 1.3 ova shed/rat) groups. However, the range in ovulation rates was considerably larger in middle-aged (0–16 ova/rat) than young (7–15 ova/rat) animals.

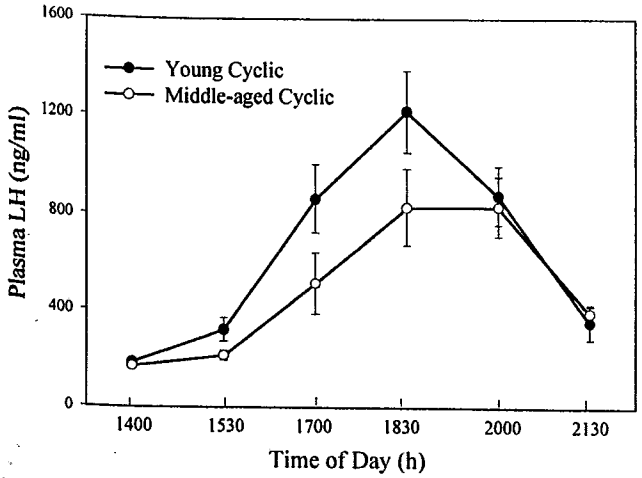

Figure 1. Proestrous LH surge profiles in young (closed circles; n = 8) and middle-aged (open circles; n = 12) regularly cyclic female rats. Serial blood samples were obtained through indwelling jugular catheters from 1400 hr to 2130 hr proestrus for LH determinations. Data represent the mean ± SEM for each group at each time point.

Correlation between LH Surge Magnitudes and Ovulation Rates in Young and Middle-Aged Cyclic Rats. While no significant difference in mean ovulation rate was observed between young and middle-aged rats when analyzed as groups, it was noted that some individual middle-aged females exhibited low ovulation rates or anovulation, despite the occurrence of a detectable LH surge (a rise in LH greater than twice the baseline value). When LH surge profiles for individual animals were compared with the same animals' ovulation rates (see representative animals in Fig. 2), it appeared that middle-aged rats with low ovulation rates tended to exhibit low preovulatory LH values. To confirm this observation, Pearson Product Moment analysis was performed, revealing a significant correlation between the ovulation rate and peak LH surge value

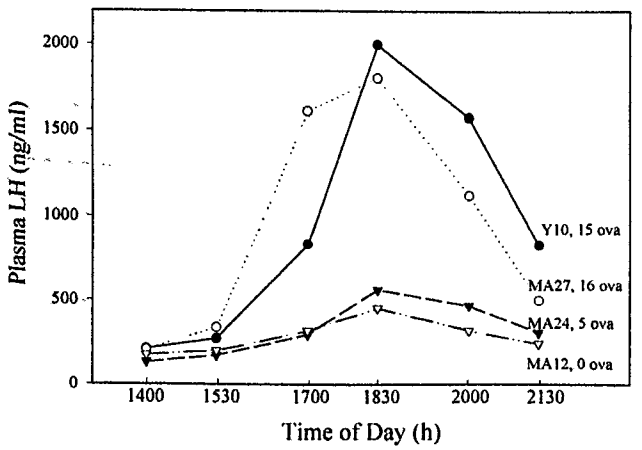

Figure 2. Representative comparisons of LH surge profiles and ovulation rates in individual young and middle-aged female rats. Young rats (as in Y10) tended to have high LH surge levels and high ovulation rates. Some middle-aged rats (such as MA18) also had high LH surges with high ovulation rates, while other middle-aged females (such as MA24 and MA12) had markedly attenuated LH surges, with few or no ovulations.

in each animal (Fig. 3; r = 0.635, $P < 0.005$; n = 20 rats). These findings indicate a direct relationship between the magnitude of the proestrous LH surge and the numbers of follicles rupturing in response to gonadotropin stimulation.

Relationship between Decreased LH Surge Magnitudes in Middle-Aged Female Rats and Cell-Specific Expression of Ovarian tPA mRNA. In view of the observed relationship between LH surge magnitude and ovulatory function, we next examined the impact of attenuated LH surges on the induction of tPA mRNA levels during the periovulatory period. The pattern of the proestrous LH surge was characterized in separate groups of young and middle-aged cyclic rats as described above. From these same animals, ovaries were collected at 2300 hr proestrus, 0200 hr estrus, or 0500 hr estrus for analysis of tPA expression. Figure 4 depicts the patterns of plasma LH observed in young cyclic rats with normal LH surges, and in middle-aged females that displayed attenuated LH surges (rats with peak LH surges <50% of that seen in young rats; n = 6 rats/group). In these same females, tPA expression was analyzed by *in situ* hybridization analysis. Prior to the onset of the LH surge (1400 hr proestrus) tPA message was detected in thecal-interstitial cells and in oocytes, but not in granulosa cells of developing follicles of ovaries from young rats (Fig. 5A). Similar cell-specific patterns of tPA expression were observed in middle-aged females on proestrous afternoon. At 0200 hr estrus granulosa cells of preovulatory follicles from young rats expressed very high levels of tPA mRNA (Fig. 5B). In contrast, a smaller induction of tPA expression was observed in granulosa cells of preovulatory follicles from middle-aged rats with attenuated LH surges (Fig. 5C).

To further determine the temporal pattern of tPA expression in young and middle-aged rats, hybridization signals over the granulosa layer of preovulatory follicles were quantified by a computerized image analysis system, and expressed as the numbers of silver grains per granulosa area

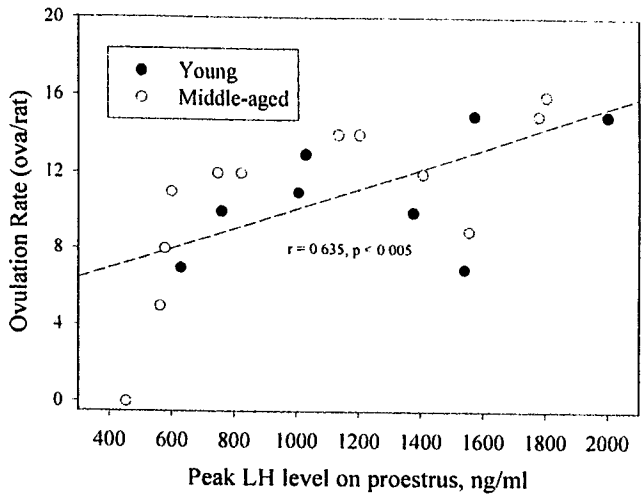

Figure 3. Correlation between ovulation rate (ova shed/rat) and peak proestrous LH level in young (closed circles) and middle-aged (open circles) female rats (n = 20).

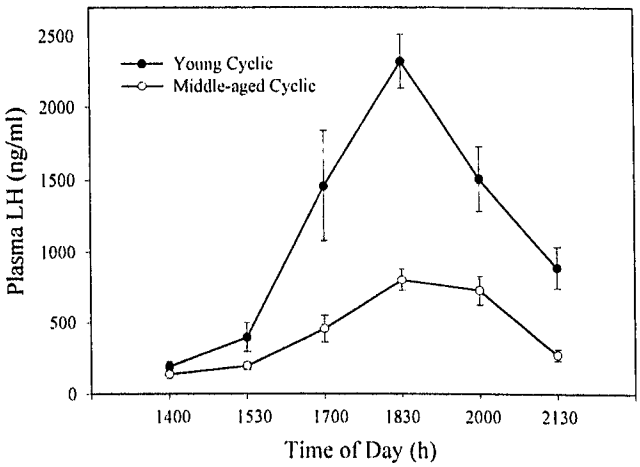

Figure 4. Proestrous LH surge profiles in young (closed circles) and middle-aged females with attenuated LH surges (open circles) prior to collection of ovaries for tPA mRNA analysis. Serial blood samples were obtained through indwelling jugular catheters between 1400 hr and 2130 hr proestrus for LH measurements (n = 6/group).

(Fig. 6; n = 4–7 follicles/group/time point). This analysis confirmed that tPA message levels were undetectable in granulosa cells of preovulatory follicles at 1400 hr proestrus, but increased slightly at 2300 hr proestrus, after the LH surge had taken place. A major increase in tPA mRNA levels (over 200-fold) was observed in granulosa cells of preovulatory follicles at 0200 hr in young rats. In middle-aged females with attenuated LH surges, tPA message also increased in granulosa cells of preovulatory follicles by 2300 hr proestrus. However, tPA mRNA levels at 0200 h estrus were significantly lower ($P < 0.01$) in middle-aged than young rats, and there was no significant increase in tPA expression at this time point in the older animals. By 0500 hr estrus, tPA expression was markedly decreased in freshly ovulated follicles of young rats, and in persisting preovulatory follicles of middle-aged females.

Discussion

Reproductive aging in rodents is associated with alterations in the neuroendocrine regulation of gonadotropin secretion (27–29) and in ovarian functions such as follicular development, steroidogenesis, and ovulation (8, 30, 31). A major challenge in understanding reproductive senescence is elucidating the changing interactions between the aging neuroendocrine system and the aging ovary. While the effects of age on altered neuroendocrine regulation of the proestrous LH surge have been well characterized, the immediate consequences of attenuated LH release on ovarian functions have received little attention. The present findings indicate that age-related declines in the proestrous LH surge result in decreased numbers of rupturing follicles or the complete absence of ovulation, depending upon the degree of attenuation. Furthermore, middle-aged rats with attenuated LH surges exhibit decreased expression of the proteolytic enzyme tPA, indicating insufficient gonadotropin stimulation to elicit this component of the ovulatory pro-

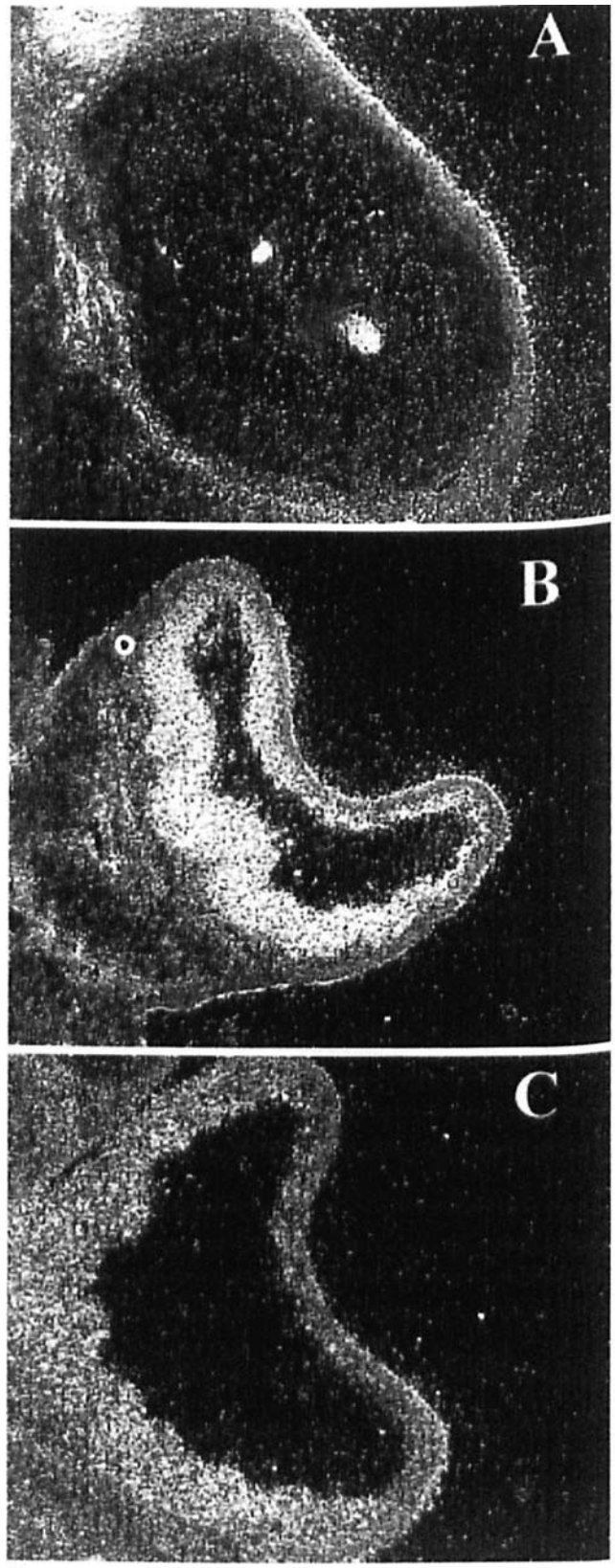

Figure 5. *In situ* hybridization analysis of tPA mRNA levels in preovulatory follicles from ovaries of young female rats at 1400 hr proestrus (A) and 0200 hr estrus (B) and from a middle-aged rat ovary at 0200 hr estrus (C). Ovaries were photographed under dark field microscopy.

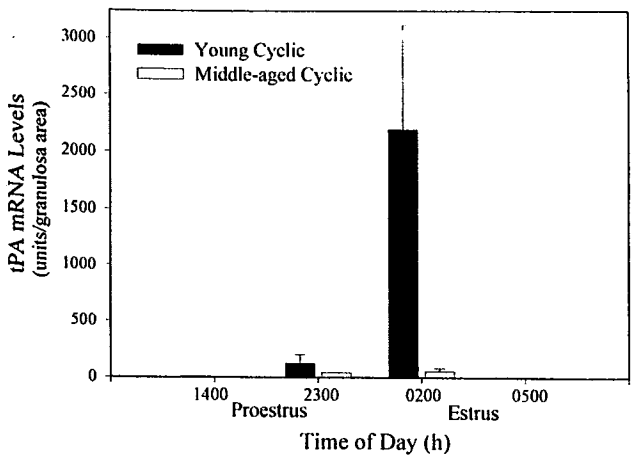

Figure 6. Analysis of tPA mRNA levels in granulosa cells of preovulatory follicles from young (closed bars) and middle-aged (open bars) rats during the periovulatory period. Hybridization signals over the granulosa layer were quantitated using a computerized digital image analysis system, and expressed as the density of silver grains/area of granulosa (n = 4–5 follicles/group/time point).

cess. Thus, age-related changes in the neuroendocrine regulation of the proestrous LH surge not only serve as markers of reproductive aging but may also immediately contribute to decreased ovulation rates and/or anovulation.

Aging populations exhibit a high degree of heterogeneity, in that not all animals exhibit signs of aging at the same rate. Thus, some but not all regularly cyclic middle-aged rats display attenuated LH surges at a given age (5). Similarly, while the mean ovulation rate was not significantly reduced in middle-aged as compared to young rats, some middle-aged females displayed few or no ovulating ova. Statistical comparison of LH surge magnitudes and ovulation rates in individual middle-aged and young rats revealed a significant correlation between LH surge magnitude and numbers of ova shed, such that middle-aged rats with attenuated LH surges exhibited decreased numbers of rupturing follicles. The relationship between attenuated LH surges and impaired ovulation in middle-aged rats may be causal, indicating insufficient gonadotropin stimulation for maximal induction of ovulation. In this regard, previous studies have reported dose-dependent effects of gonadotropins on ovulatory function (20, 31–33). Furthermore, our recent findings (33) indicate that ovulatory sensitivity to hCG stimulation is not decreased in middle-aged regularly cyclic females. Together, these observations suggest that age-related declines in proestrous LH surge levels are causally related to impaired ovulatory function in middle-aged cyclic rats.

The pattern of ovarian tPA mRNA expression during the periovulatory period in spontaneously ovulating adult rats has not, to our knowledge, been previously described. We observed that tPA was expressed in thecal-interstitial cells and oocytes of developing and preovulatory follicles, similar to results obtained by *in situ* hybridization analysis of ovaries from immature rats treated with exogenous gonadotropins (34). Consistent with immunocytochemical studies of adult cyclic rats (35), tPA message levels were low in granulosa cells of preovulatory follicles prior to the LH surge, but were markedly increased following LH stimulation and peaked just prior to ovulation. The time interval between the peak of the proestrous LH surge and peak tPA message levels is also consistent with the interval required for hCG-induced tPA expression in immature rats (34). As observed in immature, gonadotropin-treated rats (34), the induction of tPA message was minimal in cumulus cells of preovulatory follicles following gonadotropin stimulation, presumably reflecting the absence of LH receptor expression in this cell type (34, 36).

Previous studies have demonstrated that while the numbers of preovulatory follicles are similar in young and middle-aged rats, persisting preovulatory follicles are evident in middle-aged ovaries on the morning of estrus (30). Our present findings demonstrate that in middle-aged females with attenuated proestrous LH surges, the induction of granulosa cell tPA expression was markedly less than that observed in young animals. In particular, the large rise in tPA expression observed in preovulatory follicles of young rats just prior to ovulation (0200 hr estrus) was absent in most preovulatory follicles of middle-aged animals. These findings indicate an impaired induction of this proteolytic pathway in ovaries of middle-aged rats, presumably due to insufficient gonadotropin stimulation. Since decreased tPA expression is associated with the failure of preovulatory follicles to ovulate (our observations, and reference 30), altered expression of tPA (and potentially other proteases) in middle-aged rats may contribute to age-related declines in ovulatory function. Furthermore, these findings reiterate a role of tPA in mediating gonadotropin-induced ovulation. Further studies are required to determine whether other components of the multiple pathways mediating gonadotropin-induced follicle rupture are also influenced by altered neuroendocrine regulation of gonadotropin secretion during aging.

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