Ovarian Steroidogenic Responsiveness to Exogenous Gonadotropin Stimulation in Young and Middle-Aged Female Rats (44543)

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Abstract. **Reproductive aging in the female rat is associated with gradual declines in LH secretion and ovarian progesterone (P) production. This study examined whether the influences of aging on P levels reflect decreased ovarian responsiveness to gonadotropin stimulation, as opposed to changes in gonadotropin release. Young and middle-aged regularly cyclic female rats received sodium pentobarbital to block endogenous proestrous luteinizing hormone (LH) surges, followed by administration of various doses of human chorionic gonadotropin (hCG). Similar treatments were performed in middle-aged acyclic persistent-estrous (PE) females. Injection of hCG resulted in equivalent plasma hCG levels in each treatment group. At the lowest hCG dose tested, a significant rise in plasma P levels was observed in middle-aged cyclic rats, but not in young cyclic or middle-aged PE females. This unexpected finding may reflect accelerated follicular development in middle-aged cyclic females, as suggested by a previous study. At the intermediate dose, young and middle-aged cyclic but not PE rats displayed significantly increased P in response to hCG. At the highest dose tested, all three groups of rats displayed increased P levels after hCG stimulation. However, P concentrations were significantly lower in middle-aged PE than regularly cyclic females. Northern and slot blot hybridization analyses revealed that ovarian mRNA levels for cytochrome P450 side-chain cleavage, the rate-limiting enzyme in P synthesis, were markedly reduced in PE rats following hCG stimulation. These findings indicate that ovarian responsiveness to gonadotropin stimulation is impaired in middle-aged PE, but not regularly cyclic rats, and suggest influences of cycle status on the biochemical and molecular mechanisms regulating ovarian steroid production. Furthermore, these findings reveal that attenuated P production in middle-aged proestrous rats is due to attenuated preovulatory LH surges, rather than decreased ovarian sensitivity to LH.** [P.S.E.B.M. 2000, Vol 224:285–291]

Reproductive aging in both human and rodent species is characterized by increased incidences of irregular ovulatory cycles (1–4) and decreased fertility (5–7). These changes in ovulatory and reproductive functions are associated with declines in the numbers of ovarian follicles (8, 9) and altered patterns of ovarian and pituitary hormone secretion (3, 10–14). We have recently reported that ovarian ovulatory responsiveness to gonadotropin stimulation is similar in young and middle-aged cyclic rats, but markedly impaired in middle-aged acyclic, persistent-estrous (PE) females (15). To examine further the effects of aging on ovarian functions, the present study examined ovarian steroidogenic response to human chorionic gonadotropin (hCG) in young and middle-aged cyclic and middle-aged PE rats.

A hallmark of reproductive aging in cyclic middle-aged rats is the decline in proestrous LH surge magnitude (11–14). Although young rats display large proestrous LH surges, some middle-aged females display markedly attenuated proestrous LH release. Attenuated LH surges in middle-aged cyclic rats appear to result, at least in part, from decreased positive feedback responsiveness to the stimulatory effects of estradiol (E2) on LH release (16, 17). However, previous studies have also demonstrated a decline

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in proestrous P levels in middle-aged cyclic females (12, 18). The proestrous P surge is important in facilitating and maintaining LH surge magnitudes (19–21). Thus, changes in ovarian P production in middle-aged proestrous rats may contribute to age-related declines in LH secretion. The proestrous P surge is itself induced by the preovulatory surge of LH (22, 23), associated with increased expression of the rate-limiting steroidogenic enzyme, cytochrome P450 side-chain cleavage (P450scc) (24–27). Due to these reciprocal interactions, it is not clear if attenuated proestrous LH surges in middle-aged rats cause decreased P production, and/or if decreased ovarian response to LH contributes to attenuated plasma LH levels.

After the loss of regular estrous cycles, middle-aged rats exhibit irregular cycles, followed by the anovulatory PE state. PE females secrete moderate levels of plasma E2, associated with follicular development, but persistently low plasma P (1, 28). PE females fail to exhibit spontaneous LH surges and ovulation, consistent with the lack of positive feedback response to estrogen (16, 17). Interestingly, studies have shown that caging and mating with fertile males stimulates large and rapid increases in LH release in some PE females (29, 30). Moreover, this mating-induced LH surge is preceded by a large increase in plasma P (30). Recent studies also indicate that PE rats with short histories of acyclicity also display LH surges in response to P administration (31). These findings indicate that increased P levels in PE rats can elicit an LH surge. However, P production in PE rats remains chronically low, associated with low plasma LH. Therefore, alterations in P production in PE rats may also contribute to the neuroendocrine decline seen in these aging females.

As is evident from the above, the maintenance of female reproductive function is dependent upon complex feedback interactions between the ovary and neuroendocrine system. This study examined the influence of reproductive aging on ovarian steroidogenic responsiveness to gonadotropin stimulation.

Materials and Methods

Experimental Animals. Young (3–4-month-old) virgin and middle-aged (8–9-month-old) retired breeder Long-Evans female rats (Charles River Laboratories, Portage, MA) were housed in standard vivarium facilities. Temperature (24°–26°C) and lighting schedule (lights on 0500–1900 hr daily) were maintained throughout this study, with food and drinking water available *ad libitum*.

Estrous cycle patterns of these rats were determined by examination of daily vaginal smears. Only females exhibiting at least three consecutive 4-day cycles were considered regularly cyclic. Middle-aged retired breeders that had displayed at least 15 consecutive days of cornified vaginal cytology were considered to be in the anovulatory PE state. Young regularly cyclic, middle-aged regularly cyclic, and middle-aged PE rats were used in this study. Procedures involving the use of vertebrate animals were approved by the Institutional Animal Care and Use Committee, California State University, Los Angeles.

Experimental Procedures. Young and middle-aged regularly cyclic rats on diestrus Day 2, and PE females that displayed at least 15 consecutive days of persistent vaginal cornification received intrajugular catheters under ether anesthesia between 1700 and 1800 hr, similar to that previously described (32). Following an overnight recovery, cyclic animals with predominantly nucleated vaginal cytology were considered to be proestrous. At 1330 hr, proestrous rats were injected with sodium pentobarbital (Nembutal; 4.0 mg/100 g body wt sc) to block the endogenous preovulatory gonadotropin surges (15, 33, 34). For comparison, PE females were also injected with Nembutal to maintain standard treatments for all subjects. At 1600 hr, a baseline blood sample (0.4 ml) was taken *via* the catheter into a heparinized syringe, and human chorionic gonadotropin (hCG) (Sigma-Aldrich, St. Louis, MO) was then injected iv at 2.5, 5.0, or 10 mIU/g body wt (n = 5–9 rats/group). Subsequent blood samples (0.4 ml) were similarly collected at 5, 15, and 60 min following hCG treatment, and then at 90-min intervals until 2300 hr for measurements of plasma LH, hCG, and P. Plasma was collected by immediate centrifugation of blood samples, and stored at −20°C until hormone levels were determined by radioimmunoassay (RIA).

Following blood sampling, ovaries were collected at 2300 hr to examine the relationship between plasma P levels and ovarian P450scc expression. Ovaries were placed in a sterile microcentrifuge tube and frozen immediately in a dry-ice/ethanol bath and stored at −80°C. Total ovarian RNA was extracted using the single-step guanidinium thiocynate (GTC) method (35). Tissues were homogenized in the presence of GTC using a Biospec Tissue Tearor (Racine, WI). The RNA in the homogenate was phenol/chloroform-extracted and ethanol-precipitated. The pelleted RNA was resuspended in autoclaved double-deionized water. RNA concentrations were determined by absorbance measurements using a UV spectrophotometer. The RNA was stored at −80°C until further analysis.

Nucleic Acid Probe for mRNA Analysis. A P450scc cDNA was previously derived from bases 626–1167 of the published rat P450scc cDNA sequence (25, 36) and inserted into the plasmid vector pBSK (Stratagene, La Jolla, CA). The construct was linearized with the restriction enzyme *HindIII*, and a ^{32}P-labeled P450scc antisense cRNA probe was synthesized with T3 RNA polymerase, using an *in vitro* transcription system (Promega, Madison, WI). This riboprobe was used in Northern and slot blot hybridizations to determine ovarian P450scc mRNA levels.

Northern and Slot Blot Hybridization Analysis. For Northern blot analysis, 5-μg samples of total ovarian RNA were fractionated on 1% denaturing agarose gels, and then transferred overnight to nitrocellulose filters (Schleicher & Schuell, Keene, NH) by capillary action in 20× SSC buffer(3.0 *M* NaCl, 0.94 *M* sodium citrate). Slot blots were prepared by directly applying 5 μg of total RNA to nitro-

cellulose filters *via* a slot blot apparatus. RNA samples were then cross-linked to filters using an ultraviolet crosslinker (Stratagene, La Jolla, CA). Filters were hybridized with a ^{32}P-labeled P450scc cRNA probe in 50% formamide, 0.25 mg/ml herring sperm DNA, 2.5× Denhardt's, 0.001 *M* EDTA, and 0.5 mg/ml of yeast tRNA at 65°C overnight. Blots were subsequently washed under highly stringent conditions to remove nonspecific hybridization. Thereafter, filters were used to expose x-ray film (Kodak X-OMAT AR, Eastman Kodak, Rochester, NY) at −80°C, followed by development and digital imaging analysis.

RIAs for P, LH, and hCG. Circulating plasma P levels were measured by specific P RIA. Steroids were separated from plasma protein and clotting factors by extraction with ethyl ether. Purified samples were then subjected to polarity-based Celite column chromatography to isolate P fractions (1, 37). Subsequently, RIA was performed using a specific antibody to P as previously described (1, 38). Plasma LH levels were measured by a rat LH double-antibody RIA, using reagents provided by the National Institutes of Health, as previously described (39). Plasma hCG values were determined by a commercial hCG kit (Coat-a-Count, DPC, Los Angeles, CA).

Data Analysis. The intensity of P450scc hybridization signals was quantitated using the Scion ImagePC digital image analysis program (Scion Corporation, Frederick, MD). Data obtained from analysis of blots were expressed as relative P450scc mRNA levels per ovary.

Statistical comparisons of plasma P, LH, and hCG levels between young, middle-aged cyclic, and middle-aged PE rats were performed using two-way analysis of variance (ANOVA), whereas differences in P450scc mRNA levels between groups were analyzed using one-way analysis of variance. Analysis of variance was followed by the Student-Newman-Keuls test to determine differences between each time point and/or group. A confidence level of $P < 0.05$ was considered statistically significant. All data values are presented as mean ± SEM.

Results

Circulating Plasma LH and hCG Concentrations in Young Cyclic, Middle-Aged Cyclic, and Middle-Aged Persistent Estrous Rats Following Nembutal and hCG Treatments. Young and middle-aged regularly cyclic rats received Nembutal at 1330 hr on proestrus to block the endogenous preovulatory gonadotropin surges. Subsequently, various doses of hCG were injected at 1600 hr to mimic the endogenous LH surge, allowing assessment of steroidogenic responsiveness to gonadotropin stimulation. Nembutal treatment prevented the proestrous LH surge in 96% of rats studied, and only those animals with successfully blocked LH surges were included in subsequent data analyses. Middle-aged PE females were also treated with Nembutal to eliminate experimental discrepancies between groups. In all animals, plasma hCG concentrations increased from undetectable levels prior to treatment to maximal values 5 min following hCG injection (Fig. 1). Thereafter, plasma hCG declined gradually over the next 7 hr, approaching basal levels. Treatment of rats with increasing doses of hCG (2.5, 5.0, and 10.0 mIU/g body wt) resulted in increasing plasma hCG levels. There were no statistically significant differences in the circulating concentrations of hCG between young cyclic, middle-aged cyclic, and PE rats within each hCG dose. These results demonstrated that all three groups of animals received similar exposure to hCG stimulation at the different doses tested.

Circulating Plasma Concentrations of Progesterone in Response to hCG Stimulation. To examine ovarian steroidogenic responsiveness to gonadotropin stimulation, circulating P levels were determined in animals treated with the three different doses of hCG. Previous studies demonstrated that administration of Nembutal on proestrus effectively blocks the proestrous rise in P (40–42). Thus, significant increases in P levels following hCG stimulation in Nembutal-treated rats presumably reflect ovarian responsiveness to exogenous gonadotropin stimulation. Surprisingly, at the lowest dose tested (2.5 mIU hCG/g body wt) plasma P concentrations increased significantly ($P < 0.05$) only in middle-aged cyclic females, beginning 60 min (1700 hr) following hCG injection (Fig. 2, top panel), and remaining elevated until at least 2300 hr. In contrast, under the same experimental conditions, hCG administration failed to elicit a statistically significant rise in P levels in young cyclic and PE females. These results indicated that middle-aged cyclic rats have a higher steroidogenic sensitivity to gonadotropin stimulation than young cyclic and anovulatory PE animals, which do not show a significant increase in P when treated with 2.5 mIU/g body wt of hCG.

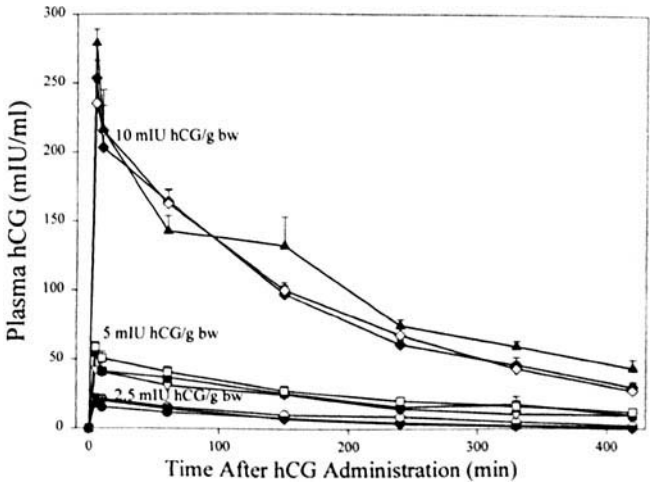

Figure 1. Levels of circulating hCG in young cyclic, middle-aged cyclic, and middle-aged PE rats treated with 2.5, 5, or 10 mIU/g body wt hCG at 1600 hr. There were no significant differences in the circulating concentrations of hCG between groups within each hCG dose ($n = 5$–9 rats per group). Legend: *2.5 mIU hCG/g body wt dose*: young cyclic (filled circles), middle-aged cyclic (open circles), middle-aged PE (filled triangles); *5.0 mIU hCG/g body wt dose*: young cyclic (open triangles), middle-aged cyclic (filled squares), middle-aged PE (open squares); *10.0 mIU hCG/g body wt dose*: young cyclic (filled diamonds), middle-aged cyclic (open diamonds), middle-aged PE (filled triangles).

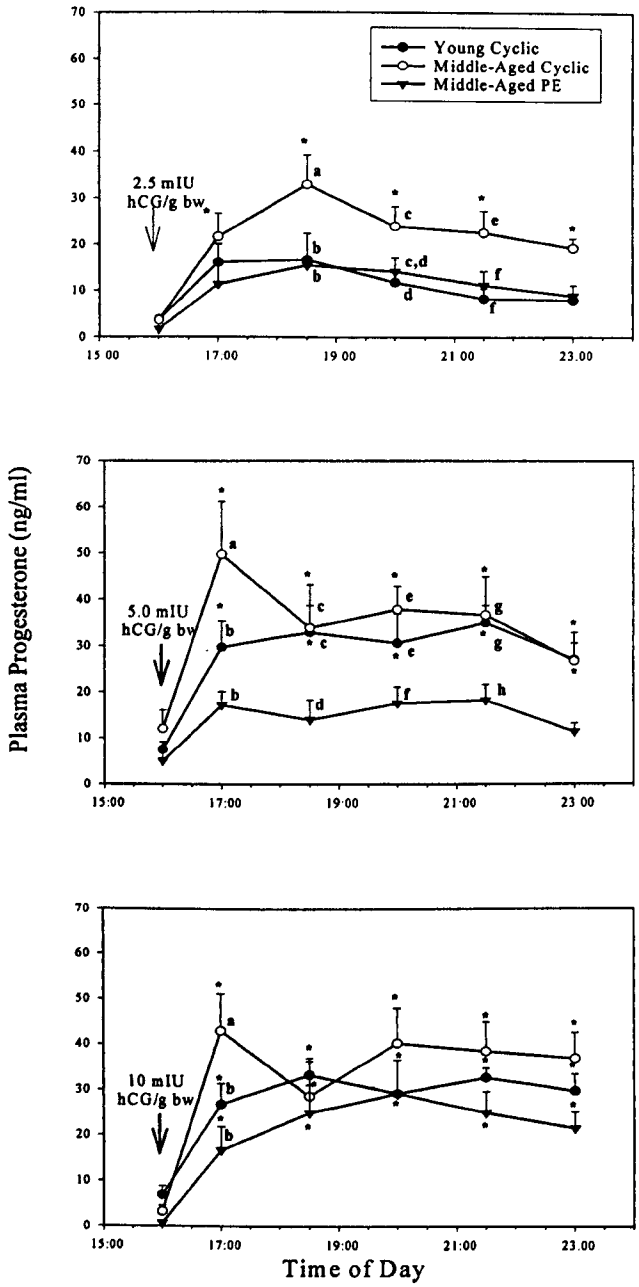

Figure 2. Plasma P levels following administration of 2.5 mIU hCG/g body wt (top panel), 5.0 mIU hCG/g body wt (middle panel), and 10.0 mIU hCG/g body wt (bottom panel) to young cyclic, middle-aged cyclic, and middle-aged PE rats (n = 5–9 rats/group). Asterisks indicate a significant rise in P level compared with baseline (before hCG injection). At each time point, symbols with differing letters indicate statistically significant differences in P levels between groups at that time point ($P < 0.05$).

At the intermediate hCG dose tested (5.0 mIU hCG/g body wt), plasma P concentrations increased significantly ($P < 0.05$) in young cyclic females within 60 min after hCG injection (1700 hr) (Fig. 2, middle panel). P levels remained nearly constant in this group throughout the remaining sampling period. Similarly, middle-aged cyclic rats also exhibited a significant increase in P secretion within 60 min of hCG treatment, and circulating P levels remained elevated until at least 2300 hr proestrus. In contrast, middle-aged PE rats did not display a statistically significant rise in P levels following hCG administration. These results indicated that this intermediate gonadotropin dose was sufficient to induce P secretion in young and middle-aged cyclic rats, but not PE females, indicating decreased ovarian sensitivity to gonadotropin stimulation in these aging animals.

At the highest dose of hCG tested (10 mIU/g body wt), we observed a significant increase in P production in all three groups within 60 min following hCG administration, and plasma P levels remained elevated throughout the evening (Fig. 2, bottom panel). This was the only dose in which a statistically significant response to hCG was observed in PE rats. In addition, the levels of hCG-stimulated P in middle-aged PE females were significantly lower than those observed in middle-aged cyclic rats at this dose ($P < 0.05$). These findings demonstrated that the ovaries of middle-aged PE rats are capable of producing increased levels of P in response to hCG, but that relatively high levels of gonadotropin stimulation are required to elicit P release.

Analysis of the mean hCG-stimulated P level for each group revealed that at the 2.5 mIU/g body wt dose of hCG, the average P level after hCG treatment was significantly higher in middle-aged cyclic than in young or middle-aged PE rats (Fig. 3) ($P < 0.05$). At the 5 mIU/g body wt dose, mean P levels were significantly higher in young and middle-aged cyclic than PE females. At the 10 mIU/g body wt dose, the mean P level in PE females was similar to that in young cyclic animals, but significantly less than that in middle-aged cyclic rats ($P < 0.05$). Together, these results confirmed that the steroidogenic responsiveness to gonadotropin stimulation is highest in middle-aged cyclic rats, but lowest in middle-aged PE females.

Ovarian Expression of P450scc in Young and Middle-Aged Female Rats. To examine factors contributing to altered steroidogenic responses during reproductive

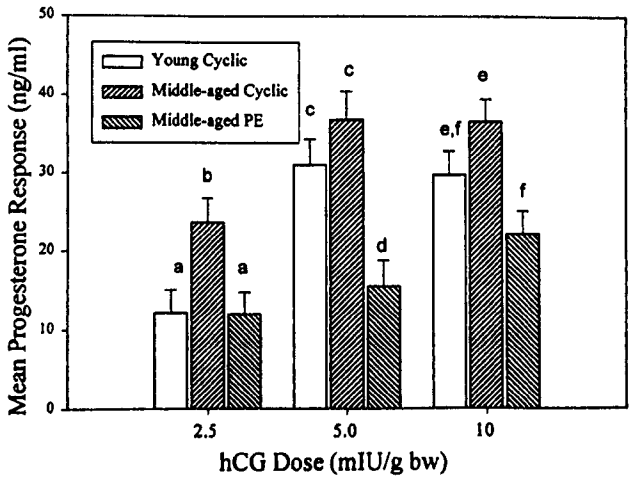

Figure 3. Mean P levels following hCG administration in young and middle-aged female rats. At each dose, the average P level following hCG treatment was calculated for each group. Within each dose, different letters above bars indicate significant differences between groups ($P < 0.05$) (n = 5–9 rats/group).

aging, we determined ovarian steady-state levels of cytochrome P450scc mRNA in hCG-treated rats. Northern blot hybridization analysis of ovarian RNA with a ^{32}P-labeled rat P450scc probe revealed a 1.9-kb transcript corresponding to the rat cytochrome P450scc mRNA, as previously reported (26) (Fig. 4). Furthermore, it appeared that the hybridization signal was less intense in ovarian extracts of middle-aged PE than in young and middle-aged cyclic females following treatment with 5 mIU hCG/g body wt (Fig. 4). More quantitative slot blot hybridization analysis (Fig. 5) confirmed that cytochrome P450scc mRNA levels were significantly lower in ovaries of PE females compared with those in young and middle-aged cyclic rats at all three hCG doses tested ($P < 0.05$; $n = 5$–8 ovaries/group), indicating decreased expression of this rate-limiting steroidogenic enzyme in anovulatory females.

Discussion

Age-related declines in reproductive function reflect changes in neuroendocrine and ovarian functions. This study evaluated whether decreased P levels in aging rats reflect changes in ovarian sensitivity to gonadotropin stimulation. The findings indicated, perhaps surprisingly, that middle-aged cyclic rats have increased ovarian sensitivity to gonadotropin stimulation, compared with young cyclic ani-

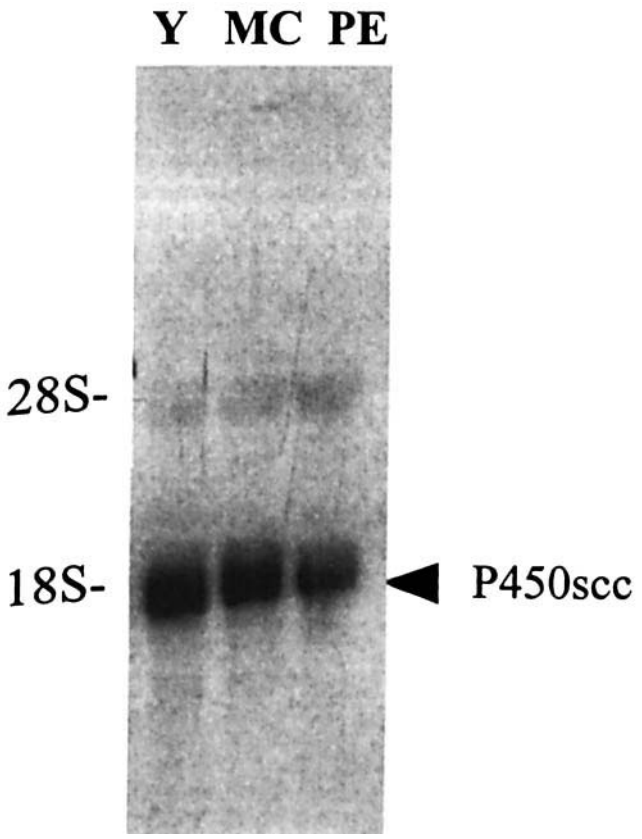

Figure 4. Northern blot analysis of ovarian P450scc mRNA levels in young cyclic (Y), middle-aged cyclic (MC), and middle-aged PE rats. Nembutal-treated rats were injected with hCG (5 mIU hCG/g body wt), followed by blood sampling as described above. Ovaries were then collected at 2300 hr, and Northern blot analysis was performed with a ^{32}P-labeled rat P450scc cRNA probe. Exposure to x-ray film revealed a 1.9-kb transcript corresponding to the rat cytochrome P450scc mRNA.

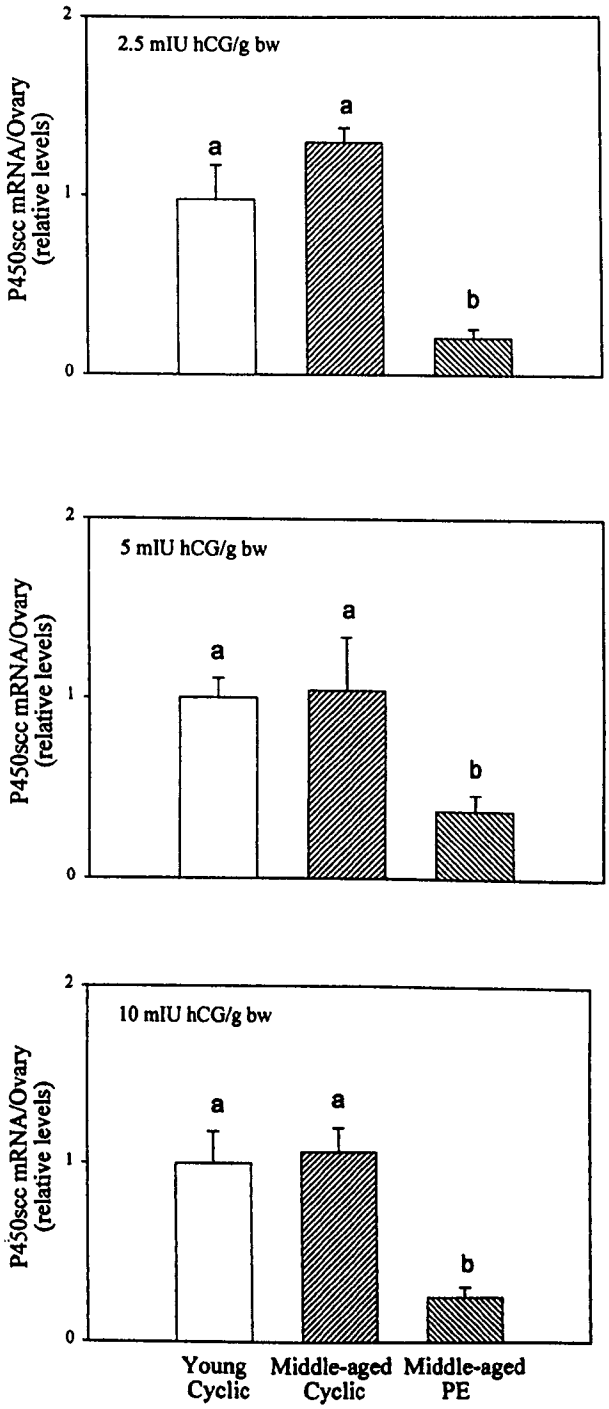

Figure 5. Slot blot hybridization analysis of ovarian P450scc mRNA levels in young and middle-aged rats following treatment with 2.5 mIU hCG/g body wt (top panel), 5 mIU hCG/g body wt (middle panel), and 10 mIU hCG/g body wt (bottom panel). Nembutal-treated rats were injected with hCG, followed by blood sampling as described above. Ovaries were collected at 2300 hr, and slot blot analysis was performed with a ^{32}P-labeled rat P450scc cRNA probe. Data are expressed as P450scc mRNA levels/ovary (n = 5–6/group). P450scc message levels were significantly lower in PE than young and middle-aged cyclic rats at each treatment dose tested.

mals. However, a decline in ovarian steroidogenic responsiveness to LH/hCG stimulation is observed once middle-aged females enter the acyclic PE state. Furthermore, impaired steroidogenic response in PE rats is associated with decreased levels of ovarian cytochrome P450scc expression, suggesting effects of cycle status on the biochemical and molecular mechanisms mediating steroid synthesis.

Whereas many studies have focused on age-related changes in hormone secretion during aging, less information is available regarding the effects of age on target cell responsiveness to hormone stimulation. Our recent report indicates that ovulatory responsiveness to hCG in middle-aged cyclic rats is equal to that in young rats, with middle-aged cyclic rats having similar ovulation rates after hCG administration (15). However, that study did not address possible changes in steroidogenic sensitivity to LH/hCG stimulation in middle-aged cyclic females. One previous report indicates decreased circulating estradiol levels in premenopausal women after treatment with human menopausal gonadotropins, associated with decreased follicular growth during the follicular phase of the menstrual cycle (43). However, that report did not examine the effects of aging on gonadotropin-stimulated P production. The present study demonstrated that treatment of young and middle-aged regularly cyclic rats with increasing doses of hCG results in the dose-dependent stimulation of P production. Surprisingly, our results reveal that ovarian steroidogenic sensitivity to hCG is higher in middle-aged cyclic than in young cyclic rats, and lowest in middle-aged PE females. Such findings could perhaps be anticipated by previous studies indicating that follicular development is accelerated in middle-aged cyclic rats. Lerner *et al.* (44) demonstrated a 2- to 4-fold increase in the proportion of large follicles in middle-aged cyclic compared with young animals. In addition, middle-aged cyclic rats had a greater number of follicles with high E2 concentrations compared with young females. Our findings confirmed that preovulatory follicles of middle-aged cyclic rats are functionally more mature than those in young rats. Furthermore, our results indicated that decreased P production in middle-aged proestrous females (12, 18) is not due to decreased ovarian responsiveness to gonadotropin stimulation.

The relationship between attenuated proestrous LH surges and decreased proestrous P levels in middle-aged cyclic rats has been unclear. Since the proestrous P surge facilitates LH release, it was conceivable that attenuated LH surges resulted from insufficient P production, due to decreased ovarian response to gonadotropin stimulation. Alternatively, since the proestrous P surge is dependent upon the LH surge, decreased proestrous P levels could be a result, rather than cause, of attenuated LH surges. Perhaps the most significant implication of the present study is that since middle-aged cyclic rats do not display impaired steroidogenic sensitivity to gonadotropins, decreased P surge levels in the middle-aged proestrous rat presumably result from attenuated LH surge magnitudes. This result indicates an immediate impact of decreased LH surges on P synthesis, and reveals that attenuated LH surges in middle-aged rats are a cause, rather than result, of decreased P production on proestrus.

Once female rats enter the PE state, they fail to experience spontaneous LH surges, despite elevated E2 levels, and become chronically anovulatory (1, 28). It was not previously clear if chronically low P levels in PE rats solely reflect impaired LH secretion, and/or decreased responsiveness to gonadotropin stimulation. Our recent findings demonstrated that despite continued follicular development in the PE ovary, ovulatory responsiveness to hCG stimulation in PE animals declines markedly compared with young and middle-aged cyclic females (15). The current study indicated that steroidogenic responsiveness to gonadotropin stimulation is also compromised in PE rats. Although this diminished response presumably reflects a decreased number of developing follicles in the PE ovary, potential changes in gonadotropin receptor expression and/or signal transduction in PE rats may also take place. Together with the findings of Anzalone *et al.* (15), these studies demonstrated impaired ovarian responsiveness to gonadotropin stimulation in PE females.

Decreased P production in PE rats was associated with lower message levels for cytochrome P450scc. This observation is consistent with the role of P450scc as the rate-limiting enzyme in P production, and suggests that age-related changes in the regulated expression of this gene product contribute to altered steroid production during aging. Since ovarian P450scc activity and expression after the LH surge is localized to theca cells of all developing follicles as well as granulosa cells of preovulatory follicles (26), declines in P450scc expression in PE rats may reflect a decreased number of healthy developing as well as preovulatory follicles in PE animals (45). In addition, decreased P450scc expression in PE rats may also reflect the effects of cycle status upon the mechanisms regulating P450scc expression, resulting in less P450scc mRNA and enzyme per preovulatory follicle and/or granulosa area following hCG stimulation. Further studies using *in situ* hybridization techniques are required to reveal the effects of aging on the cellular localization and regulation of ovarian P450scc.

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