Ovarian Carbonyl Reductase Delayed Onset of Persistent Estrus in the Offspring of Parathyroidectomized Mother Rats

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Received April 19, 1995 Accepted June 5, 1995

ABSTRACT—We investigated the effects of maternal parathyroidectomy on day 5 of pregnancy on the ovarian carbonyl reductase (CR) in the offspring of rats. Changes in the ovarian CR of the offspring were examined at 4 and 8 months of age. In 8-month-old female offspring, the ovarian weight and the ovarian CR activity were significantly higher in rats from parathyroidectomized mothers than in rats from sham-operated mothers, and the offspring of the parathyroidectomized mothers showed the regular 4-day estrous cycles at 8 months of age, while the offspring of the sham-operated mothers were in the state of persistent estrus. Furthermore, intense immunostaining was found in the theca interna cells and the interstitial gland cells in the ovary of rats from the parathyroidectomized mothers, whereas in the ovary of the age-matched normal rats, immunostaining was faint. These results suggest that the maternal parathyroidectomy affect the activity and localization of CR in the ovary of female offspring.

Keywords: Carbonyl reductase, Parathyroidectomy in pregnancy, Ovary, Persistent estrus

Fujii (1) has reported for the first time that the characteristic response of serum calcium to parathyroid removal in the first (F1) generation rats from mothers parathyroidectomized on day 5 of pregnancy (Px-F1 rats) is inherited into the third and fourth generations. Moreover, Fujii and Yamamoto (2) have reported that female Px rats show the regular 4-day estrous cycle over 12 months of age, whereas rats from sham-operated mothers (normal rats) show persistent estrus and demonstrated that the aging process of the hypothalamo-pituitary-ovarian axis in Px rats is delayed. On the other hand, Caplan and Beguin (3) have reported that in patients with hypoparathyroidism, requirements for calcium and vitamin D decline significantly during lactation.

We have already purified and characterized carbonyl reductases (CRs, EC 1.1.1.184) from rat ovary (4) and testis (5) and human testis (6). CR catalyzes the NADPH-dependent reduction of a variety of endogenous and xenobiotic carbonyl compounds to the corresponding alcohols, and it is known as prostaglandin 9-keto reductase. Indeed, CRs from rat ovary and testis have both prostaglandin 9-keto reductase and prostaglandin 15-keto reductase activity with comparatively low Km (4, 5). The ovarian CR has been demonstrated to be induced by luteinizing hormone/human chorionic gonadotropin (LH/hCG), and the induction is potentiated by estrogen in immature (7–9) and mature (10, 11) rats. In the present study, we investigated changes in the ovarian CR activity and its content in Px-F1 rats.

MATERIALS AND METHODS

Animals

Virgin female Wistar-Imamichi strain rats (8-weeks-old) were purchased from the Institute for Animal Reproduction (Ibaragi) and housed in group cages under controlled conditions of light (14 hr on, 10 hr off) and temperature (24°C). Food and water were always available. Rats were made pregnant by mating with fertile males, and pregnancy was confirmed by the presence of vaginal sperm on day 0. On day 5 of pregnancy, the rats were parathyroidectomized under ether anesthesia, and sham-operations were performed on the control animals. Rats born to parathyroidectomized and sham-operated mothers (Px-F1 rats and normal rats, respectively) were weaned at 21 days of age, and female and male rats were isolated at 5 weeks of age. Virgin female offspring were used for the experiments at 4 and 8 months of age.

Chemicals

Nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) was obtained from Oriental Yeast Co.
Table 1. Changes in body weight, ovarian and adrenal weights, and ovarian CR concentration in relation to ovulation

| Group | Body wt. (g) | Ovarian wt. (mg) | No. of ova | CR conc. (pg/mg) | Adrenal wt. (mg) |
|-------|--------------|------------------|------------|------------------|-----------------|
| 4 months |             |                  |            |                  |                  |
| Normal | 287.0 ± 7.73 | 144.2 ± 12.11    | 13 ± 0.8   | 31.5 ± 4.08      | 68.0 ± 4.68     |
| Px-F1  | 297.0 ± 10.41 | 193.7 ± 14.33*  | 12 ± 0.9   | 27.1 ± 1.04      | 67.0 ± 3.61     |
| 8 months |             |                  |            |                  |                  |
| Normal | 340.2 ± 18.92†| 77.0 ± 6.80†    | 0          | 10.8 ± 1.74"     | 64.0 ± 2.47     |
| Px-F1  | 314.8 ± 6.85 | 134.2 ± 1.77‡%,* | 11 ± 1.3  | 20.0 ± 1.49%,*   | 54.8 ± 1.24%,*  |

Rats were sacrificed at 4 and 8 months of age. Each value represents the mean ± S.E. of 5 rats.

*P < 0.05, **P < 0.01, significantly different from normal rats. †P < 0.05, ‡P < 0.01, significantly different from 4-month-old rats.

Immunochemical methods

Enzyme preparation and enzyme assay

Rats were sacrificed by decapitation, and the ovaries of each rat at 4 and 8 months of age were isolated. Each tissue was homogenized in ice-cold 10 mM phosphate buffer (pH 6.5) containing 1 mM dithiothreitol, 0.5 mM EDTA and 0.154 M KCl, and the tissue homogenate was centrifuged at 10,5000 x g for 60 min at 4°C. The resulting supernatant (cytosolic fraction) was used for the assay of enzyme activity as a crude enzyme preparation, and the reductase activity was photometrically assayed in a 1-ml incubation mixture consisting of 100 mM phosphate buffer (pH 6.5), cytosolic fraction, substrate solution and 0.1 mM NADPH for 3 min at 37°C in a Hitachi U-3200 Spectrophotometer (Tokyo). One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 μmol of NADPH per min at 340 nm under the assay conditions.

Protein concentration in the ovarian cytosol was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard.

Enzyme quantitation

The quantitation of CR in the ovarian cytosol was performed by the Western blot-peroxidase anti-peroxidase (PAP) assay using anti-rat ovarian CR2 antiserum as described previously (8). Briefly, ovarian cytosolic protein (1 μg) was subjected to SDS-PAGE (10% gel) and blotted onto a polyvinylidene difluoride membrane obtained from Atto Co. (Tokyo). Enzyme protein on the membrane was visualized, and the amount of the enzyme protein was measured by a densitometer (CS9000; Shimadzu Co., Kyoto) using purified rat ovarian CR2 as a standard.

Immunohistochemical methods

The ovaries were isolated quickly, immersed immediately in 100 mM phosphate-buffered (pH 7.4) 4% paraformaldehyde, and fixed for 24 hr. After fixation, the samples were sequentially dehydrated in 70–100% alcohol and then embedded in paraffin. The paraffin blocks were cut into 4-μm-thick slices, and the immunostaining with anti-rat ovarian CR2 antiserum was carried out by the avidin-biotin peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Lab., Burlingame, CA, USA) as previously described (5, 7). Control sections were treated with normal rabbit serum.

Statistical analyses

All enzyme activities are expressed as mU per mg cytosolic protein and all CR contents, as μg per mg cytosolic protein. Duncan's multiple range test was used for comparison between groups, with a P value of 0.05 or less considered to indicate a significant difference.

RESULTS

At 4 months of age, both normal and Px-F1 rats showed the regular 4-day estrous cycles. At 8 months of age, Px-F1 rats had the regular cyclicity and ovulation occurred, while normal rats transferred to acyclicity. The ovarian weight of Px-F1 rats were higher than that of normal rats at both 4 and 8 months of age. The adrenal weight of Px-F1 rats was 85.6% that of the normal rats at 8 months of age.

The ovarian CR concentration was significantly decreased at 8 months of age in both normal and Px-F1 rats as compared with that at 4 months of age (Table 1). However, the ovarian CR concentration in 8-month-old Px-F1 rats was about twofold that in age-matched normal
Fig. 1. Changes in ovarian CR activity towards four substrates in 4- and 8-month-old normal and Px-F1 rats. □ normal, □ Px-F1. Each column shows the mean ± S.E. of 5 rats. *P < 0.05, **P < 0.01; significantly different from normal rats. #P < 0.05, ##P < 0.01, ###P < 0.001, significantly different from 4-month-old rats.

Fig. 2. Western blot analysis of ovarian CR in normal and Px-Fl rats at 8 months of age. S1 and S2, purified ovarian CR2 (9.1 and 18.2 ng, respectively); lanes 1 to 5, ovarian cytosol of normal rats; lanes 6 to 10, ovarian cytosol of Px-F1 rats. Lanes 1 to 10 each contained 1 µg of cytosolic protein.
rats, although there was no significant difference between normal and Px-F1 rats at 4 months of age.

Figure 1 shows the ovarian CR activity of normal and Px-F1 rats at 4 and 8 months of age. The reductase activities towards four substrates at 8 months of age was significantly decreased to 26–66% of the levels at 4 months of age in normal rats, and in particular, the decrease in 4BP reducing activity was most significant (20.93 ± 1.36 mU/mg at 4 months of age and 5.51 ± 1.33 mU/mg at 8 months of age). In 8-month-old Px-F1 rats, the magnitude of the decrease (15–58%) in the reductase activities towards four substrates was smaller than in normal rats, although the activities were significantly decreased, except for PNBA reductase activity. At 4 months of age, PNAP reductase activity was significantly greater in Px-F1 rats than in normal rats. Furthermore, the enzyme activities in Px-F1 rats at 8 months of age were 1.3 to 2.2-fold of those in normal rats. The 4BP reductase activity, which well reflects the rat ovarian CR activity (4,10), was 1.8-fold higher in Px-F1 rats than in normal rats.

Fig. 3. Correlation between CR activity and CR concentration in normal and Px-F1 rats. The enzyme activity expresses 4BP reductase activity (mU/mg protein), and the enzyme concentration is expressed as µg/mg cytosolic protein. • 4-month-old normal rats, ○ 8-month-old normal rats, □ 4-month-old Px-F1 rats, □ 8-month-old Px-F1 rats. Five animals for each.

Fig. 4. Typical immunohistochemical localization of ovarian CR in normal and Px-F1 rats at 8 months of age. A: normal rat ovary, B: Px-F1 rat ovary. G, granulosa cells; T, theca interna cells; I, interstitial gland cells. Magnification × 25.
The higher concentration of the ovarian CR in 8-month-old Px-F1 rats were also demonstrated by Western blot analysis as shown in Fig. 2 (lanes 6 to 10). The relationship between the enzyme activity (4BP reductase activity) and the enzyme concentration at 4 and 8 months of age of normal and Px-F1 rats is plotted in Fig. 3. The enzyme activity was well correlated with the enzyme concentration (P<0.01). This indicates that changes in the CR concentration reflect the enzyme activity in the ovary.

Figure 4 shows the localization of ovarian CR by immunohistochemistry. The interstitial gland cells in 8-month-old rat ovary were faintly stained, and no positive reactivity in the theca interna cells was observed (Fig. 4A), whereas intense immunostaining was found in the theca interna cells and in the interstitial gland cells in 8-month-old Px-F1 rat ovary (Fig. 4B).

DISCUSSION

The decline in female reproductive functions of rats with aging is characterized by constant cornified vaginal smears, the presence of well-developed follicles in the ovary, and the lack of preovulatory LH surge (13). A complex process of the hypothalamo-pituitary-ovarian axis including the pituitary response to luteinizing hormone releasing hormone (LHRH) and the ovarian response to gonadotropins is involved in the decline in reproductive functions (14). However, the detailed mechanism of reproductive senescence in each component of the axis is not clear.

Recently, it is indicated that calcium ion plays important roles in reproductive functions such as hormone release (15, 16). We have found that exogenous calcium has biphasic effects on testicular CR activity in 3-week-old rats but not in older animals (17). The results that Px offspring delays the aging process of hypothalamo-pituitary-ovarian axis described by Fujii and Yamamoto (2) are of interest. In the present study, changes in the ovarian CR activities and concentrations with aging as one of the parameters of reproductive functions were determined. Furthermore, we have already demonstrated the ovarian CR is a LH dependent enzyme in immature (7-9) and mature rats (10, 11).

All of the normal rats showed persistent estrus and a significant decrease in the ovarian weight at 8 months of age. We have recently reported that consecutive treatments with estrogens, estradiol and synthetic estrogens, inhibit both ovulation and ovarian CR activity in mature cycling rats, and that hCG treatment restores the inhibitory effects of estrogens (18). These results in mature cycling rats are associated with those observed in aged rats, including the decrease in ovarian CR activity. Accordingly, it is concluded that the elevated estrogen levels may cause not only the inhibition of the pre-ovulatory LH surge but also the decline in the expression of the ovarian CR.

Fujii (1) has reported that a decrease in the hypocalcemic response in mature female Px-F1 rats is observed after unilateral or bilateral parathyroidectomy, while serum calcium levels in normal rats at the same age showed a rapid decrease after the operation, and suggested that Px-F1 rats acquire altered regulation in serum calcium during embryonic development within hypocalcemic mothers that are parathyroidectomized on day 5 of pregnancy. Garel (19) has described that large amounts of calcium are transferred across the placenta from the maternal to the fetal circulation during pregnancy. It is well known that one of the factors that play an important role in calcium metabolism is parathyroid hormone (PTH). The parathyroidectomy-induced maternal hypocalcemia may result in a reduced supply of calcium to the fetal circulation. Low levels of fetal calcium are thought to increase fetal plasma PTH levels. Indeed, as Bagnoli et al. (20) have reported that in newborns, calcium and PTH concentrations were negatively correlated, fetal tissues in parathyroidectomized mothers may be exposed to large amounts of PTH secreted from the fetal parathyroid glands. The results that the ovarian CR activity and its concentration in Px-F1 rats, which had regular cyclicity, were higher than those in normal rats, which had persistent estrus, indicate that the maternal parathyroidectomy may positively influence the hypothalamo-pituitary-ovarian axis in the female offspring. Furthermore, Veldhuis et al. (21) have reported that an inhibitor of intracellular calcium mobilization and calmodulin antagonists inhibit LH-stimulated progesterone production and suggested that calcium ions modulate LH action in ovarian cells in part by a calcium-calmodulin-dependent mechanism. Accordingly, the altered calcium metabolism during the fetal life may cause the delayed onset of persistent estrus in Px-F1 female rats.

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