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REGULATION OF OVARIAN CARBONYL REDUCTASE MEDIATED BY ESTROGEN RECEPTOR IN IMMATURE RATS

NORIHISA INAZU,* NIRO INABA and TETSUO SATOH†

Department of Pharmacology and Toxicology, Tokyo College of Pharmacy, Tokyo 192-03; and †Laboratory of Biochemical Pharmacology and Biotoxicology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 260, Japan

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Abstract—In the present study, the enhancing effect of synthetic estrogen on ovarian carbonyl reductase, a new prostaglandin (PG)-metabolizing enzyme, was investigated, and the antagonistic effect of antiestrogen on this enhancement was examined in immature rats. Ovarian carbonyl reductase activity towards 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (15KD-$PGF_{2\alpha}$), 4-benzoylpyridine (4BP) and menadione was determined photometrically and radiochemically, and quantitation of ovarian carbonyl reductase content was performed by Western blot analysis. Diethylstilbestrol (DES) and hexestrol (HX) enhanced the increasing effect of human chorionic gonadotropin (hCG) on ovarian carbonyl reductase activity and content when these synthetic estrogens (0.2 mg/kg) were administered for 3 days from 26 days of age, before hCG treatment. On the other hand, tamoxifen, which inhibits the binding of estradiol to the estrogen receptor, significantly prevented estradiol (E_2) from enhancing the effect of hCG on ovarian carbonyl reductase. Furthermore, the ovarian carbonyl reductase activities towards the three substrates correlated well with the ovarian carbonyl reductase content. These results indicate that ovarian carbonyl reductase in immature rats may be regulated by a specific increase in the ovarian response to luteinizing hormone mediated by estrogen receptor.

We have demonstrated that $PGF_{2\alpha}$‡ is metabolized to 13,14H_2-$PGF_{2\alpha}$ via 15-keto-$PGF_{2\alpha}$ and 15KD-$PGF_{2\alpha}$ in rat ovary [1, 2], and that 13,14H_2-$PGF_{2\alpha}$ stimulates ovarian steroidogenesis *in vitro* from pregnenolone [3]. Recently, two isozymes of the ovarian enzyme catalyzing the conversion of 15KD-$PGF_{2\alpha}$ to 13,14H_2-$PGF_{2\alpha}$ have been electrophoretically purified and characterized enzymologically [4]. These two isozymes, designated as CR1 and CR2, differed in isoelectric point, but both were confirmed to be carbonyl reductase. Carbonyl reductase (EC 1.1.1.184) is a member of the aldo/keto reductase family, like aldose reductase (EC 1.1.1.21) and aldehyde reductase (EC 1.1.1.2), and is a cytosolic monomeric oxidoreductase that catalyzes NADPH-dependent reduction of various carbonyl compounds [5]. The ovarian carbonyl reductase was shown immunohistochemically to be localized in the theca cells and the interstitial gland cells by using anti-CR2 antibody, and it was found that the enzyme activity in immature rats pretreated with estradiol (E_2) is stimulated markedly by human chorionic gonadotropin (hCG) [6]. Reich *et al.* [7] reported that luteinizing hormone-stimulated plasminogen activator activity *in vitro* in Graafian follicle is enhanced by E_2. As Harman *et al.* [8] have already suggested a role of estrogen in ovarian response to gonadotropins, it would be very interesting to examine whether or not changes in both activity and content of ovarian carbonyl reductase are mediated by estrogen receptor.

The present report presents the results of studies that were conducted in immature rats, in which it was found that pretreatment with synthetic estrogen enhanced both the activity and the content of the ovarian carbonyl reductase induced by hCG, and that the enhancement by estrogen was antagonized by antiestrogen.

MATERIALS AND METHODS

Animals. Immature female rats of the Wistar-KY strain (Japan SLC, Shizuoka, Japan) were used for the experiments. They were housed in group cages (four or five rats per cage) under controlled conditions of light (12 hr on, 12 hr off) and temperature (24°). Food and water were always freely available.

Chemicals. Diethylstilbestrol (DES), hexestrol (HX) and tamoxifen were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and hCG was from Teikoku Hormone Mfg. (Tokyo, Japan). E_2 was obtained from E. Merck (Darmstadt, F.R.G.). 4-Benzoylpyridine (4BP), menadione, dithiothreitol (DTT) and EDTA were purchased from Wako Pure Chemical Industries (Osaka, Japan), and NADPH

* Correspondence: Dr. Norihisa Inazu, Department of Pharmacology and Toxicology, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

‡ Abbreviations: PG, prostaglandin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; 15KD-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-$PGF_{2\alpha}$; 13,14H_2-$PGF_{2\alpha}$, 13,14-dihydro-$PGF_{2\alpha}$; DTT, dithiothreitol; E_2, estradiol; DES, diethylstilbestrol; HX, hexestrol; hCG, human chorionic gonadotropin; and 4BP, 4-benzoylpyridine.

was from the Oriental Yeast Co. (Osaka, Japan). [5,6,8,9,11,12,14-^{3}H]-15KD-PGF$_{2\alpha}$ (sp. act. 80 Ci/mmol) was obtained from Amersham International plc (Amersham, U.K.) and authentic 15KD-PGF$_{2\alpha}$ was from Upjohn Pharmaceuticals Ltd. (Kalamazoo, MI, U.S.A.). Authentic 13,14H$_2$-PGF$_{2\alpha}$ was a gift of the Ono Pharmaceutical Co. (Osaka, Japan). Other chemicals of reagent grade were obtained from Wako Pure Chemical Industries.

Drug treatment. DES, HX and tamoxifen were each dissolved in sesame oil, and hCG was dissolved in 0.9% saline solution. Synthetic estrogen (0.2 mg/rat) and tamoxifen (1 mg/rat) were administered s.c. daily to immature rats for 3 days from 26 days of age, and hCG (10 I.U./rat) was administered s.c. at 3:00 p.m., at 28 days of age. E_2 (10 μg/rat) was administered s.c. daily to immature rats for 3 days from 26 days of age and control animals were given (s.c.) vehicle alone. The ovaries of each rat were isolated at 9:00 a.m. at 29 days of age and homogenized in ice-cold 10 mM phosphate buffer (pH 6.5) containing 1 mM DTT, 0.5 mM EDTA and 0.154 M KCl.

Enzyme assay. The ovarian homogenate was centrifuged at 4° for 20 min at 9000 *g* in a Kubota KR/18000 (Tokyo, Japan), and the supernatant fraction obtained was used for the assay of enzyme activities as a crude enzyme preparation. The reduction of 4BP (1 mM) and menadione (0.2 mM) was determined photometrically in a total volume of 1 mL containing 100 mM phosphate buffer (pH 6.5), 0.1 mM NADPH and 0.3 mL of 9000 g supernatant; the reduction of 15KD-PGF$_{2\alpha}$ was measured radiochemically by the method described in our previous report [6].

Determination of the enzyme. The ovarian carbonyl reductase content in the 9000 *g* supernatant fraction was assayed by Western blot analysis using anti-CR2 antiserum [4]. The amount of the enzyme protein on the blot was measured by densitometry, and the standard curve was prepared by using purified CR2 in the range from 4.46 to 17.85 ng.

Statistical analysis. Experimental data are presented as the mean ± SE. For comparisons between groups, Student's *t*-test was used. Differences between groups were considered significant for P values of 0.05 or less.

RESULTS

Western blot analysis. The Western blot of purified CR2 (4.46 to 17.85 ng) in Fig. 1A gave a single band increasing in intensity with the increase in CR2 content. The laser scan of the blot generated well-defined peaks (Fig. 1B), and their areas could be used to prepare a standard curve (Fig. 1C). This

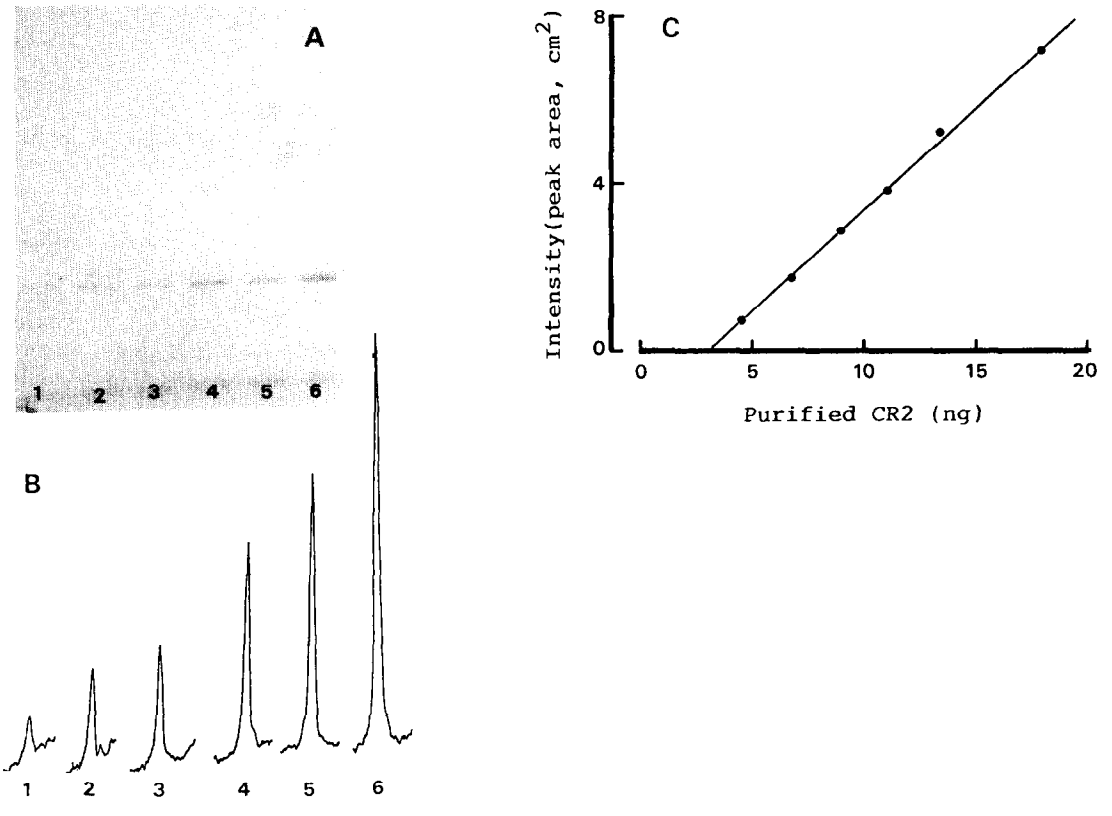

Fig. 1. Western blot analysis of purified CR2 using anti-CR2 antibody. (A) Western blot. The amounts (ng) of purified CR2 applied to the gels were as follows: lane 1, 4.46; lane 2, 6.69; lane 3, 8.93; lane 4, 11.16; lane 5, 13.39; and lane 6, 17.85. (B) Results of laser densitometry of the Western blot of purified CR2. (C) Standard curve obtained by plotting amount of CR2 applied (A) against peak area (B).

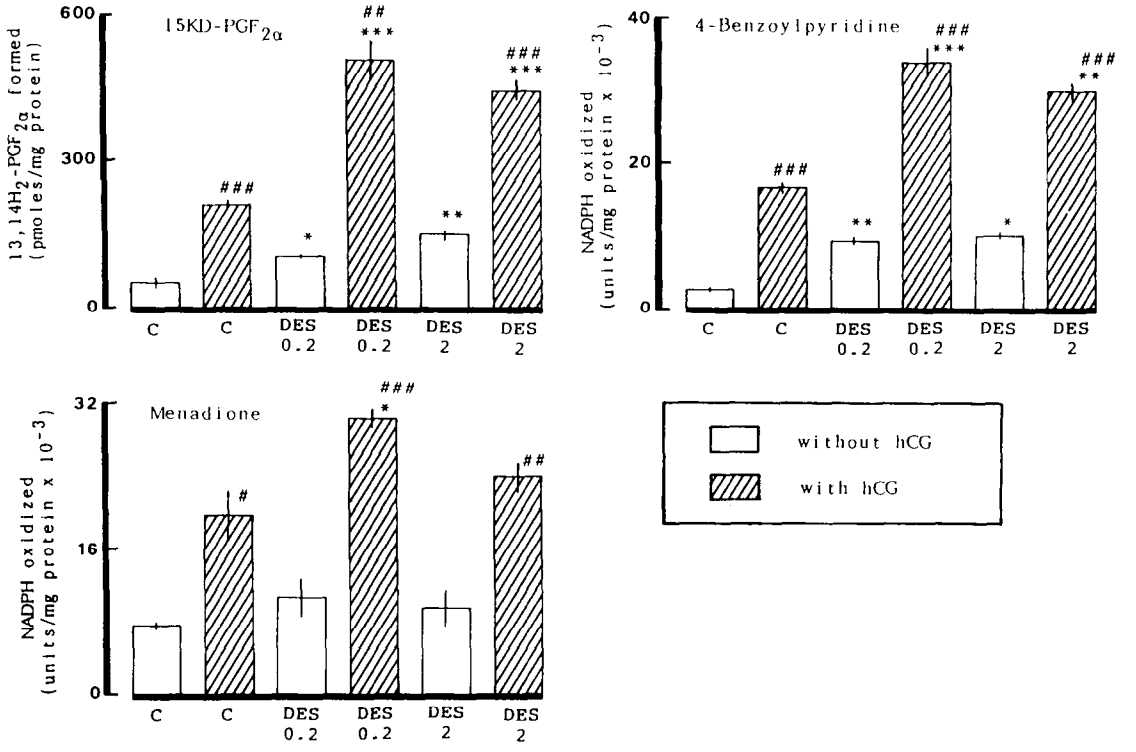

Fig. 2. Effects of co-administration of DES with hCG on the ovarian carbonyl reductase activities towards three substrates in immature rats. DES (0.2 or 2 mg/rat) was administered s.c. to immature rats for 3 days from 26 days of age, and hCG (10 I.U./rat) was given s.c. at 3:00 p.m. at 28 days of age. The ovaries were isolated at 9:00 a.m. at 29 days of age. Each column shows the mean ± SE of four to five rats. Key: C, control; DES 0.2, 0.2 mg/day diethylstilbestrol; DES 2, 2 mg/day diethylstilbestrol; and hCG, human chorionic gonadotropin. Statistical analysis: significantly different from control: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significantly different from hCG non-treated: (#) $P < 0.05$, (# #) $P < 0.01$, and (# # #) $P < 0.001$.

Western blot analysis had a limit of detection of approximately 4 ng and was linear up to 20 ng.

Effects of synthetic estrogen and hCG on ovarian carbonyl reductase. The effects of co-administration of synthetic estrogens, DES and HX, with hCG on the ovarian carbonyl reductase activities towards three substrates in immature rats were investigated (Figs. 2 and 3). The three substrates, 15KD-$PGF_{2\alpha}$, 4BP and menadione, have already been demonstrated to be specific substrates for ovarian carbonyl reductase, CR1 and CR2, in rats [4, 6].

Treatment of rats with hCG increased the reduction of 15KD-$PGF_{2\alpha}$, 4BP and menadione to approximately 3-fold over the vehicle control, but both DES and HX caused only a slight increase in the enzyme activities. The co-administration of the synthetic estrogens with hCG resulted in large increases (2- to 3-fold) in the carbonyl reductase activities towards the three substrates compared with hCG alone. The specific activities of ovarian carbonyl reductase were significantly greater in the groups where synthetic estrogens at 0.2 mg/rat were co-administered with hCG than in those where synthetic estrogens at 2 mg/rat were co-administered with hCG. It is clear that DES and HX both enhanced the increasing effect of hCG on the ovarian carbonyl reductase activity.

Figures 4 and 5 show the effects of synthetic estrogens and hCG on ovarian and uterine weights and on the ovarian enzyme content in immature rats. The ovarian weight was increased significantly by hCG treatment, and pretreatment with synthetic estrogens caused a further increase in the ovarian weight increased by hCG. The increases in the ovarian weight were dependent on the dose of the synthetic estrogens. The ovarian carbonyl reductase content was also increased by hCG treatment, and 0.2 mg/rat of synthetic estrogens markedly enhanced the action of hCG on the ovarian carbonyl reductase content. On the other hand, uterine weight was increased significantly by treatment with the synthetic estrogens, although hCG itself had no effect. Changes in the ovarian carbonyl reductase activities were in agreement with those in the ovarian carbonyl reductase content in the present study.

Effects of antiestrogen on ovarian carbonyl reductase. Whether or not tamoxifen, an anti-estrogen, could inhibit the enhancement of the increasing effect of hCG on ovarian carbonyl reductase by E_2 was investigated (Figs. 6 and 7). E_2 enhanced the activities, increased by hCG, of the ovarian carbonyl reductase towards the three substrates to about 2- or 4-fold as compared with hCG alone, whereas tamoxifen inhibited the reducing

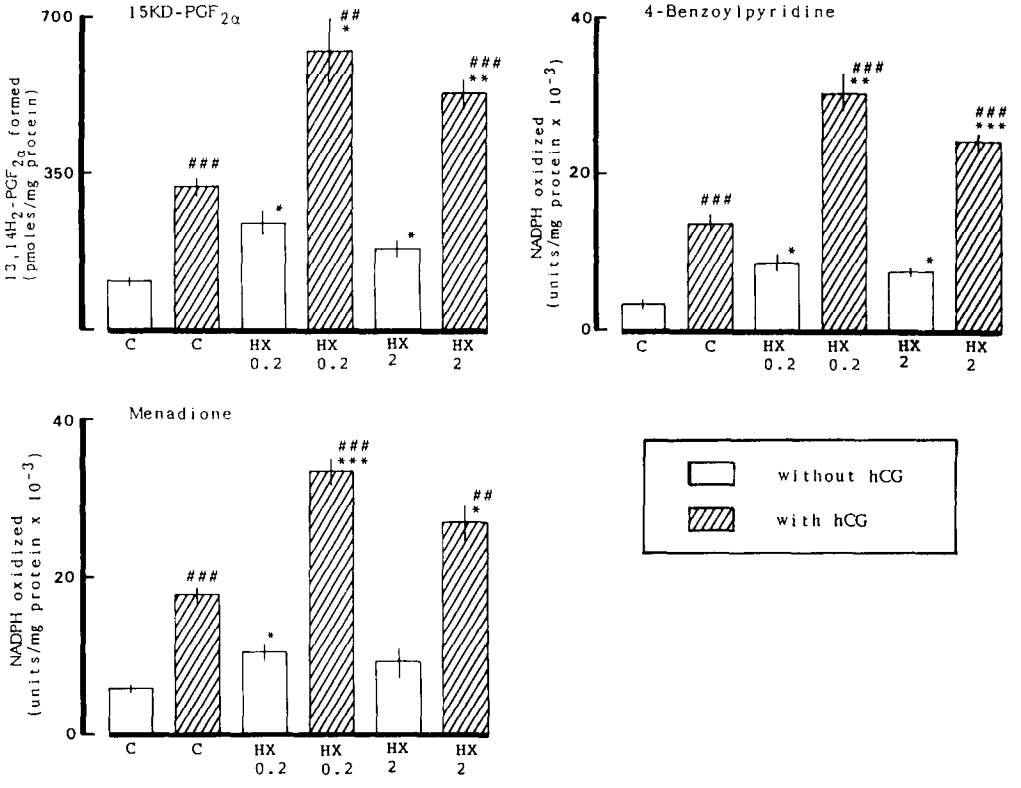
15KD-PGF2α
13,14H2-PGF2α formed (pmoles/mg protein)
700
350
4-Benzoylpyridine
NADPH oxidized (units/mg protein x 10-3)
40
20
0
Menadione
C
HX 0.2
HX 2
without hCG
with hCG

Fig. 3.

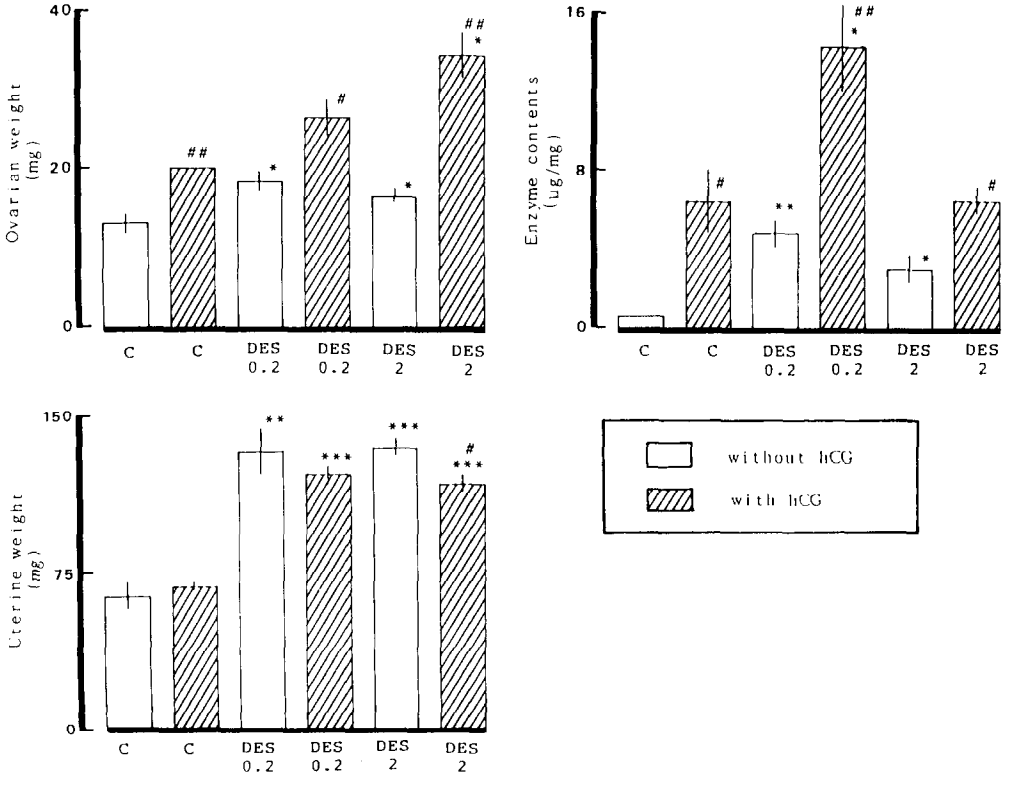
Ovarian weight (mg)
40
20
0
Enzyme contents (ug/mg)
16
8
Uterine weight (mg)
150
75
C
DES 0.2
DES 2
without hCG
with hCG

Fig. 4.

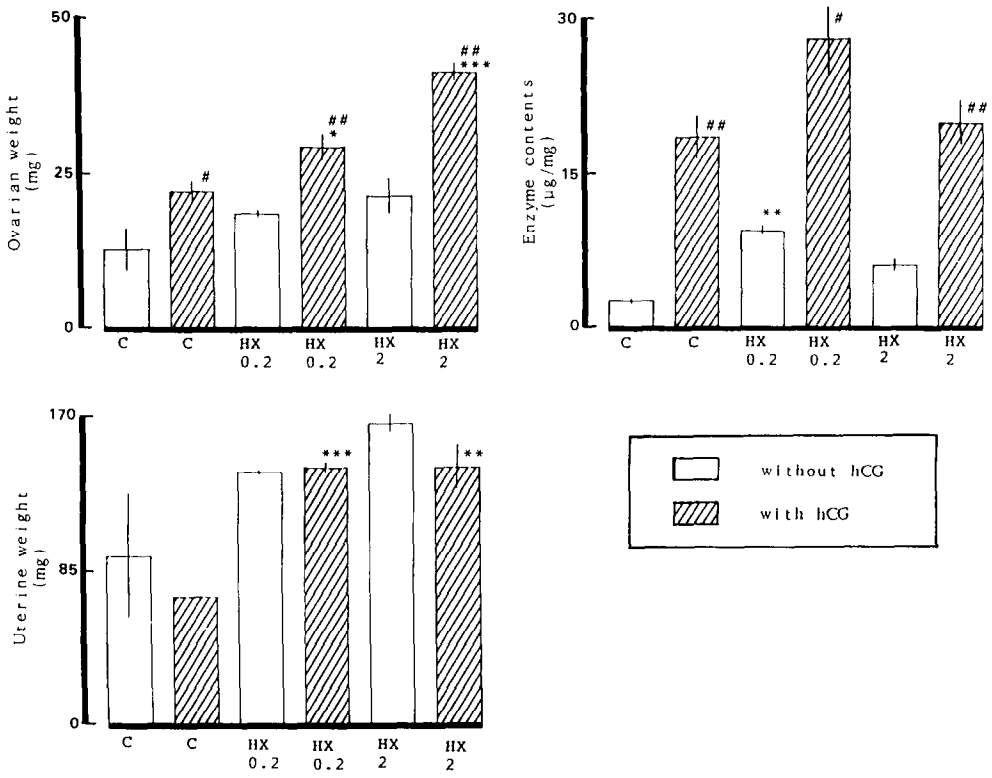

Fig. 5. Effects of co-administration of HX with hCG on the ovarian and uterine weights, and the ovarian carbonyl reductase content, in immature rats. The experimental conditions were as given in the legend of Fig. 3. Each column shows the mean ± SE of four rats. Key: C, control; HX 0.2, 0.2 mg/day hexestrol; HX 2, 2 mg/day hexestrol; and hCG, human chorionic gonadotoropin. Statistical analysis: significantly different from control: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significantly different from hCG non-treated: (#) $P < 0.05$, and (# #) $P < 0.01$.

activities toward 15KD-PGF$_{2\alpha}$, 4BP and menadione to about 56, 44 and 65% of those of the E_2 + hCG treated group, respectively, without causing any inhibition of the action of hCG alone. Furthermore, the uterine weight gain induced by E_2 was inhibited significantly by tamoxifen treatment and the ovarian weight gain and the increase in the enzyme content induced by hCG in E_2-pretreated immature rats also were reduced significantly by this antiestrogen (Fig. 7).

From these results (Figs. 2–7), it is clear that the ovarian carbonyl reductase activities towards the three substrates correlated significantly with the ovarian carbonyl reductase content.

DISCUSSION

The purpose of this study was to investigate whether or not the E_2-induced enhancement of the stimulation of ovarian carbonyl reductase by hCG may be mediated through the estrogen receptor. In the present study, DES and HX as synthetic estrogens, and tamoxifen as an antiestrogen were used. We have already reported that E_2 enhances

Fig. 3. Effects of co-administration of HX with hCG on the ovarian carbonyl reductase activities towards three substrates in immature rats. HX (0.2 or 2 mg/rat) was administered s.c. to immature rats for 3 days from 26 days of age, and hCG (10 I.U./rat) was given s.c. at 3:00 p.m. at 28 days of age. The ovaries were isolated at 9:00 a.m. at 29 days of age. Each column shows the means ± SE of four rats. Key: C, control; HX 0.2, 0.2 mg/day hexestrol; HX 2, 2 mg/day hexstrol; and hCG, human chorionic gonadotropin. Statistical analysis: significantly different from control: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significantly different from hCG non-treated: (# #) $P < 0.01$, and (# # #) $P < 0.001$.

Fig. 4. Effects of co-administration of DES with hCG on the ovarian and uterine weights, and the ovarian carbonyl reductase content, in immature rats. The experimental conditions were as given in the legend of Fig. 2. Key: C, control; DES 0.2, 0.2 mg/day diethylstilbestrol; DES 2, 2 mg/day diethylstilbestrol; and hCG, human chorionic gonadotoropin. Each column shows the mean ± SE of four to five rats. Statistical analysis: significantly different from control: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significantly different from hCG non-treated: (#) $P < 0.05$, and (# #) $P < 0.01$.

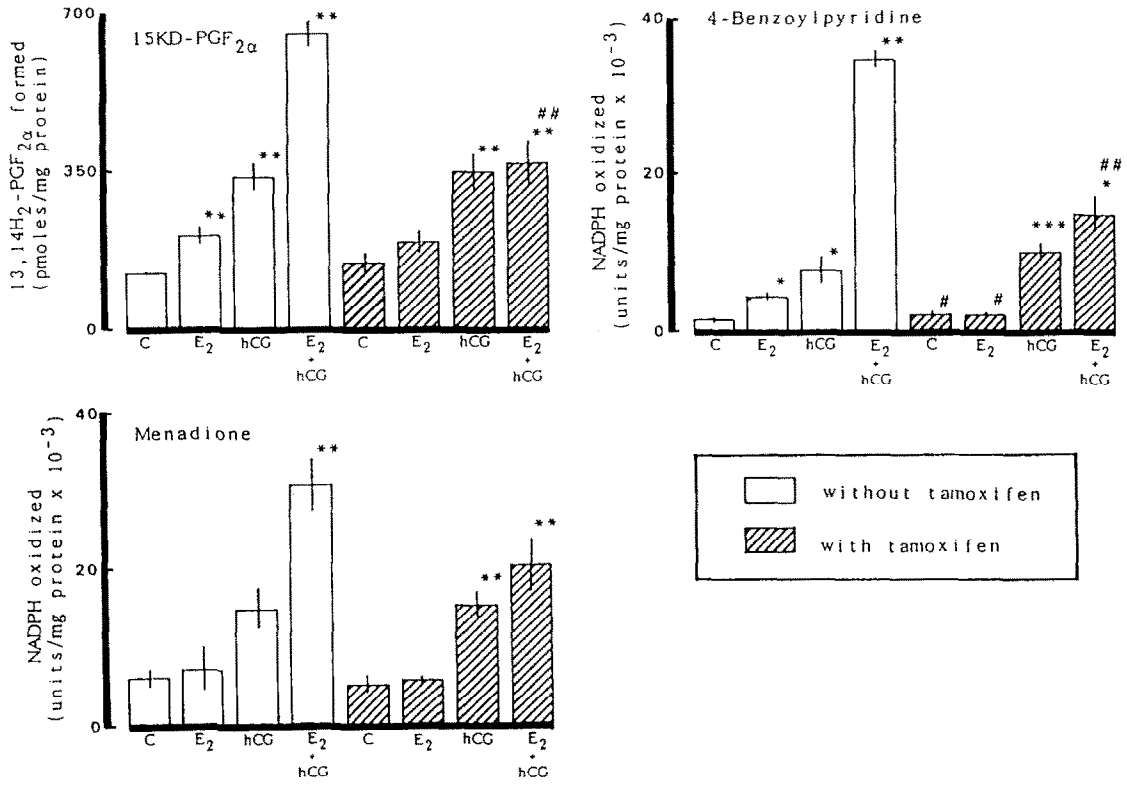

Fig. 6. Enhancement by E_2 and decrement by tamoxifen on the hCG-induced ovarian carbonyl reductase activities in immature rats. E_2 (10 mg/rat) and tamoxifen (1 mg/rat) were administered s.c. to immature rats for 3 days from 26 days of age, and hCG (10 I.U./rat) was given s.c. at 3:00 p.m. at 28 days of age. The ovaries were isolated at 9:00 a.m. at 29 days of age. Each column shows the mean ± SE of four to five rats. Key: C, control; E_2, estradiol; and hCG, human chorionic gonadotropin. Statistical analysis: significantly different from control: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significantly different from tamoxifen non-treated: (#) $P < 0.05$, and (# #) $P < 0.01$.

the increasing effect of hCG on ovarian carbonyl reductase activities, whereas progesterone and estradiol-17α do not, and that the stronger immunoreactivity to anti-CR2 antibody in E_2–hCG-treated ovary as compared with hCG-treated ovary is localized on the theca cells and the interstitial gland cells [6]. The binding capacity of luteinizing hormone/hCG in rat ovary [9, 10] and hamster ovary [11] has been reported to be much greater on the day of proestrus compared with other stages of the estrous cycle. Reiter *et al.* [12] have reported that treatment with anti-estrogen antiserum significantly inhibits both the increase in ovarian weight and the growth of ovarian follicle induced by gonadotropins, and suggested that endogenous estrogen plays an important role in the ovarian response to luteinizing hormone and follicle stimulating hormone. Saiduddin and Zassenhaus [13] studied the properties of E_2-binding protein in immature rat ovary in detail as compared with E_2 receptor in uterus, and found that the E_2-binding protein is similar to uterine E_2 receptor. We have demonstrated in the present study that DES and HX enhanced the increasing effect of hCG on both the ovarian carbonyl reductase activities and content as well as E_2, and that tamoxifen markedly inhibited the enhancement by E_2 of the ovarian carbonyl reductase activities and content, and the increase in the uterine weight induced by E_2. On the basis of these facts, the enhancement of the increasing effect of hCG on the ovarian carbonyl reductase activity by E_2 is thought to be due to a specific increase in the ovarian response to luteinizing hormone/hCG mediated by the estrogen receptor. Furthermore, the ovarian carbonyl reductase was indicated to be a luteinizing hormone-dependent enzyme which is regulated by estrogen.

Although carbonyl reductase generally catalyzes both NADP(H)-dependent reduction of the 9-keto group and oxidation of the 15-hydroxy group of PG, the K_m values for PG are high and the turnover numbers are low. Thus, some questions as to the physiological significance of carbonyl reductase in PG metabolism remain [5]. Although the K_m values of the ovarian carbonyl reductase, CR1 and CR2, for 9-keto PG were of the same order of magnitude as those of other PG-metabolizing enzymes, such as 15-hydroxyprostaglandin dehydrogenase and 9-ketoprostaglandin reductase, the K_m value for 15KD-$PGF_{2\alpha}$ (35 μM) was lower than those for other PGs (110–160 μM) [4, 14–17]. Furthermore, as the reduction of 15KD-$PGF_{2\alpha}$ in ovarian cytosol of rats was inhibited completely by anti-CR2 antibody, it was demonstrated that the ovarian carbonyl reductase predominantly contributed to the formation of

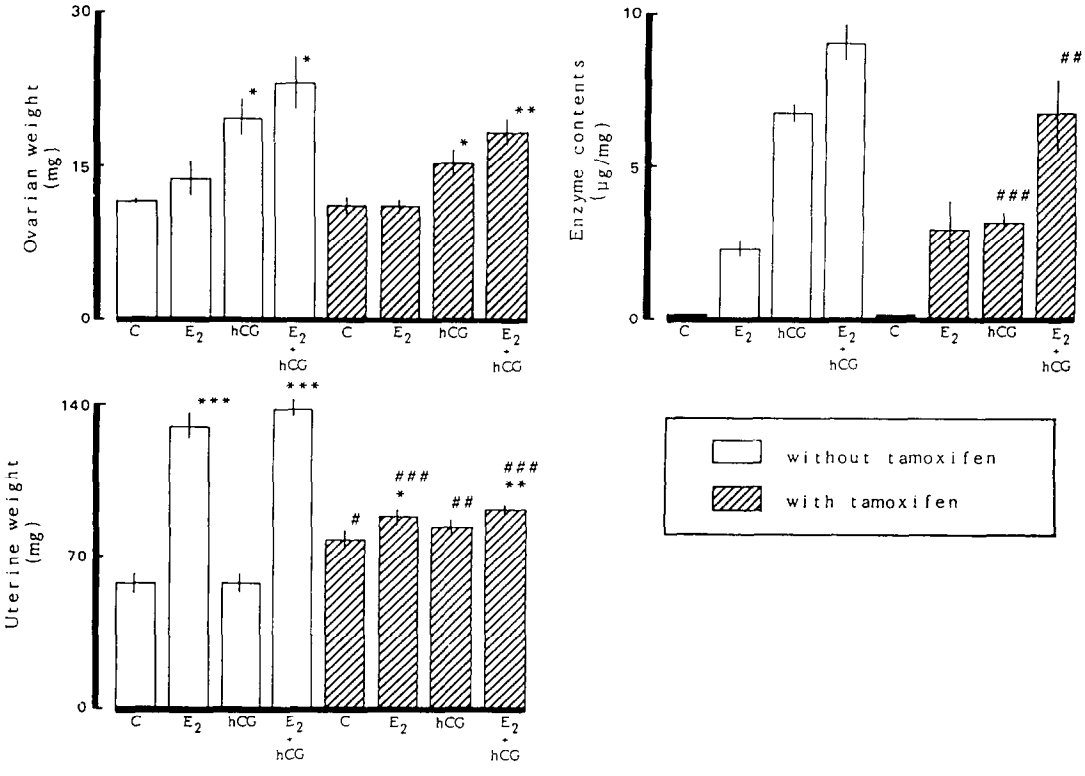

Fig. 7. Enhancement by E_2 and decrement by tamoxifen of the ovarian and uterine weights, and the ovarian carbonyl reductase content in immature rats. The experimental conditions were as given in the legend of Fig. 6. Key: C, control; E_2, estradiol; and hCG, human chorionic gonadotropin. Statistical analysis: significantly different from control: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$; significantly different from tamoxifen non-treated: (#) $P < 0.05$, (# #) $P < 0.01$, and (# # #) $P < 0.001$.

$13,14H_2$-$PGF_{2\alpha}$ from 15KD-$PGF_{2\alpha}$ in rat ovary, $13,14H_2$-$PGF_{2\alpha}$, which has stimulatory action *in vitro* on ovarian steroidogenesis in rats [3] and contractile activity *in vivo* on the uterine smooth muscle in women [18], is a biologically active metabolite of $PGF_{2\alpha}$. Changes in both the ovarian carbonyl reductase activity and content are thought to affect directly the formation and action of $13,14H_2$-$PGF_{2\alpha}$.

On the other hand, positive immunoreactivity to anti-CR2 antibody was detected in the theca cells and the interstitial gland cells, but not in the granulosa cells when *in vivo* localization of the ovarian carbonyl reductase in rat ovary was immunohistochemically investigated. Matsuda *et al.* [19] demonstrated that aromatase was localized primarily in the theca interna and the interstitial gland cells in immature and mature rat ovary by using an immunocytochemical method, and they suggested that it was reasonable to consider from a morphological viewpoint that these cells have the ability to produce estrogens. Moreover, Yoshinaga-Hirabayashi *et al.* [20] found that 17β-hydroxysteroid dehydrogenase, which is involved in the synthesis of testosterone, an important precursor of E_2, from androstenedoine, is localized in the theca interna and the interstitial gland cells. These facts indicate that both the theca interna and the interstitial gland cells have a capacity for *de novo* synthesis of E_2 from testosterone in rat ovary. It is clear that the localization of the ovarian carbonyl reductase is similar to that of aromatase and 17β-hydroxysteroid dehydrogenase, that estrogen participates in the regulation of the enzyme, and further that $13,14H_2$-$PGF_{2\alpha}$ formed by the enzyme stimulates the biosynthesis of estrogen. In rat ovary, these enzymes, including the ovarian carbonyl reductase, and endogenous factors, including $13,14H_2$-$PGF_{2\alpha}$, are strongly suggested to be implicated in ovarian function.

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