Effects of Antiestrogens on Ovarian Aldo-Keto Reductase in Relation to Ovulation in Rats

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ABSTRACT—The pharmacological effects of antiestrogens on ovarian aldo-keto reductase and ovulation were investigated in rats. The activities of reduction of 13,14-dihydro-15-keto-PGF2α, 4-benzoylpyridine and menadione in ovarian cytosol were significantly decreased by antiestrogen treatments, and the ovulation was completely inhibited. However, administration of luteinizing hormone-releasing hormone at 3:00 p.m. on the day of proestrus restored both enzyme activities and ovulation, which were inhibited by antiestrogens, to control levels. These results indicate that nonsteroidal antiestrogen inhibits the luteinizing hormone surge on the day of proestrus in 4-day cycling rats and that ovarian aldo-keto reductase may be closely involved in the ovulatory process in rats.

Nonsteroidal estrogen antagonists, such as tamoxifen and clomiphene which are estrogen antagonists of triphenylethylene derivatives, have been reported to have a variety of effects in estrogen target tissues (1–4).

Tamoxifen has been proven to be effective in the management of human breast cancer (5) and clomiphene is clinically used to induce ovulation in anovulatory women (6). It is well-recognized that antiestrogens prevent estrogens from expressing their full effects on estrogen target tissues (3, 7, 8). Estrogen is well-known to have stimulatory effects on ovarian follicle growth (7, 8), proestrus surge of LH (9) and PRL release (2, 10).

We have recently reported a new metabolic pathway for PGF2α in rat ovary (11, 12) in which PGF2α is metabolized to 13,14H2-PGF2α via 15-keto-PGF2α and 15KD-PGF2α. The carbonyl reductase (CR1, CR2) catalyzing conversion of 15KD-PGF2α to 13,14H2-PGF2α in rat ovary is a member of the aldo-keto reductase family (13) and is localized in the ovarian cytosolic fraction (14). The activities of the ovarian CRs were significantly increased after

Abbreviations used are: LH, luteinizing hormone; PRL, prolactin; PGF2α, prostaglandin F2α; 13,14H2-PGF2α, 13,14-dihydro-PGF2α; 15KD-PGF2α, 13,14-dihydro-15-keto-PGF2α; hCG, human chorionic gonadotropin; LH-RH, luteinizing hormone-releasing hormone; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MPA, medroxyprogesterone acetate; PNAP, 4-nitroacetophenone; PNBA, 4-nitrobenezaldehyde; 4BP, 4-benzoylpyridine; DTT, dithiothreitol; [3H]-15KD-PGF2α, [5,6,8,9,11,12,14-3H]-13,14-dihydro-15-keto-PGF2α.
the endogenous LH surge during the estrous cycle of rats (15) and were markedly stimulated by co-administration of estradiol and hCG in immature rats (16). We designed our experiments to characterize the pharmacological effects of four antiestrogens which are triphenylethylene derivatives and one steroidal antiestrogen on ovarian aldo-keto reductase activities towards carbonyl compounds and further investigated the effect of exogenous LH-RH on ovarian aldo-keto reductase activities inhibited by antiestrogens.

MATERIALS AND METHODS

Animals

Mature female rats of the Wistar-KY strain (7 weeks old, ca. 180 g) were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan, and they were housed in group cages (4 or 5 rats per cage) under controlled conditions of light (12 hr on, 12 hr off) and temperature (24°C). Water and food were always available. Only animals that had shown at least two normal 4-day cycles, as determined by daily vaginal smears immediately before the start of the experiment, were used.

Chemicals

NADPH was obtained from Oriental Yeast Co., Osaka, Japan. Tamoxifen citrate (trans form), clomiphene citrate (racemic compound) and nafoxidine hydrochloride were purchased from Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A. U-23469 (1,2-propanediol,3-[p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-l-naphthyl)-phenoxy]-(±)-cis-, isomer A) and MPA were kindly provided by Upjohn Pharmaceuticals, Ltd., Kalamazoo, MI, U.S.A. PNAP, PNBA, menadione, 4BP, DTT and EDTA were purchased from Wako Pure Chemical Co., Osaka, Japan; and 3H-15KD-PGF2α (sp. act. 80 Ci/mmol) purchased from Amersham Int. plc., U.K. Authentic 15KD-PGF2α was obtained from Upjohn Pharmaceuticals, Ltd.; and authentic 13,14H2-PGF2α was the generous gift of Ono Pharmaceutical Co., Ltd., Osaka, Japan. LH-RH was a gift from NIADDK, Bethesda, MD, U.S.A.

Drug treatment

Each antiestrogen was suspended in sesame oil at a concentration of 1 mg/ml, and LH-RH was dissolved in 0.9% saline solution at a concentration of 5 μg/ml.

In experiment 1, antiestrogen (1 mg/kg) was daily administered s.c. to rats for 3 days at 9:00 a.m. from the first day of diestrus. In experiment 2, antiestrogen (1 mg/kg) was singly administered s.c. to rats at 9:00 a.m. on the first day of diestrus. LH-RH (500 ng/rat) was given s.c. to rats at 3:00 p.m. on the day that proestrus was expected. Control rats were given vehicle alone. The ovaries and uterus of each rat were isolated at 18 hr after LH-RH treatment, and the presence of ova in the oviduct was determined microscopically (17).

Enzyme assay

The ovaries isolated from each rat were weighed and homogenized in 8 ml of ice-cold 10 mM phosphate buffer (pH 6.5) containing 1 mM DTT, 0.5 mM EDTA and 1.15% KCl. The ovarian homogenate was centrifuged at 4°C for 60 min at 105000 X g by a Hitachi Automatic Centrifuge Model 70P-72, and the 105000 X g supernatant (cytosolic fraction) obtained was used for the assay of enzyme activities as a crude enzyme preparation. The reduction of PNAP (1 mM), PNBA (1 mM), 4BP (1 mM) and menadione (0.2 mM), respectively, was assayed in 1 ml of incubation mixture consisting of 100 mM phosphate buffer (pH 6.5), ovarian cytosol, substrate solution and NADPH solution (final conc. 0.1 mM) for 3 min at 37°C by a Hitachi 150-20 Spectrophotometer. One unit of enzyme activity was expressed as the amount of enzyme that oxidized 1 μmole of NADPH per min at 340 nm under the assay condition. 15KD-PGF2α reducing activity was determined by a radiochemical method as previously described (14) and expressed as pmoles/mg protein/15 min of 13,14H2-PGF2α formed.

Student’s t-test was used for the statistical analysis.
RESULTS

Inhibition of consecutive treatment of antiestrogen and stimulation of LH-RH

A significant decrease in uterine weight and an inhibition of ovulation were caused by three consecutive administration of antiestrogen (1 mg/kg), although MPA did not significantly change the uterine weight. LH-RH treatment was restored the inhibition of ovulation by each antiestrogen, although the uterine weight was not changed (Table 1). However, the inhibition of ovulation by MPA was not restored by LH-RH treatment.

The ovarian weight was significantly decreased by tamoxifen and U-23469, and co-administration of tamoxifen with LH-RH restored the ovarian weight to control levels. Other antiestrogens had no effect on the ovarian weight.

Changes in ovarian aldo-keto reductase activities are shown in Figs. 1 and 2. The reduction of 15KD-PGF2α, which is not only a specific substrate for ovarian CR1 and CR2 but also an endogenous substrate in rat ovary (13), was significantly decreased to 39.2, 49.2, 62.9, 38.8 and 39.8% of the control levels by tamoxifen, clomiphene, nafoxidine, U-23469 and MPA, respectively (Fig. 1). Both the reductions of 4BP and menadione were also decreased to about 40–75% of the control levels by each antiestrogen treatment. LH-RH increased the reducing activities of 15KD-PGF2α and 4BP decreased by tamoxifen, clomiphene, nafoxidine and U-23469. These increases were agreement with restoration of ovulation. However, the reducing activities decreased by MPA were not restored by LH-RH treatment. On the other hand, no significant changes in any of the PNAP and PNBA reducing activities in ovarian cytosol were observed overall when these antiestrogens and LH-RH were administered under the present experimental conditions (Fig. 2).

Table 1. Effect of LH-RH and three consecutive treatments of antiestrogen on ovarian and uterine weights in relation to ovulation in rats

|                | Tissue weight (mg) | No. of rats | No. of ova |
|----------------|-------------------|-------------|------------|
|                | Ovary             | Uterus      |            |            |
| Control        | 69.9 ± 1.77       | 279.3 ± 6.04 | 8/8        | 12 ± 0.4   |
| LH-RH          | 75.1 ± 2.30       | 274.0 ± 5.71 | 8/8        | 10 ± 0.8   |
| Tamoxifen      | 56.0 ± 3.94**     | 242.3 ± 8.88** | 0/4   | —          |
| + LH-RH        | 72.3 ± 1.11*      | 220.5 ± 10.24** | 4/4 | 9 ± 1.1   |
| Clomiphene     | 69.3 ± 8.88       | 224.7 ± 6.36** | 0/4   | —          |
| + LH-RH        | 72.3 ± 3.71       | 208.3 ± 10.64** | 4/4 | 7 ± 2.2   |
| Nafoxidine     | 64.3 ± 3.68       | 200.0 ± 7.37** | 0/4   | —          |
| + LH-RH        | 75.0 ± 3.54       | 216.0 ± 7.22** | 4/4 | 7 ± 1.9   |
| U-23469        | 64.3 ± 0.85*      | 213.0 ± 7.91** | 0/4   | —          |
| + LH-RH        | 61.0 ± 1.29**     | 200.0 ± 3.19*** | 3/4 | 9 ± 0.4   |
| MPA            | 67.5 ± 2.36       | 256.3 ± 8.39 | 0/4       | —          |
| + LH-RH        | 63.3 ± 2.02*      | 228.3 ± 8.77** | 0/4   | —          |

Each antiestrogen (1 mg/kg) was administered s.c. at 9:00 a.m. for 3 days from the first day of diestrus, and LH-RH (500 ng/rat) was administered s.c. at 3:00 p.m. on the day of proestrus. Ovaries and uterus of each rat were isolated at 24 hr after the final treatment with each antiestrogen. Each value indicates the mean ± S.E. *P < 0.05, **P < 0.01. ***P < 0.001 vs. control; †††P < 0.01 vs. antiestrogen alone.
Fig. 1. Effect of LH-RH and three consecutive treatments of antiestrogen on reduction of 15KD-PGF$_{2\alpha}$, 4-benzoylpyridine and menadione in the ovarian cytosol of rats. Experimental conditions were as described in Table 1. Each column indicates the mean ± S.E. R control, 0 LH-RH, ® antiestrogen alone, ® antiestrogen + LH-RH. Tam., tamoxifen; Clo., clomiphene; Naf., nafoxidine; U, U-23469; MPA, medroxyprogesterone acetate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; # P < 0.05, P < 0.01, P < 0.001 vs. antiestrogen alone.

Inhibition of single treatment of antiestrogen and stimulation of LH-RH

The relationship between endogenous LH surge and single treatment with antiestrogen was examined in order to compare it with the results obtained by three consecutive treatments with antiestrogen. Table 2 shows the changes in the ovarian and uterine weights, and the occurrence of ovulation in rats when single administration of each antiestrogen on the first day of diestrus and administration of LH-RH on the day of proestrus were performed. The ovarian and uterine weights were significantly decreased by antiestrogen treat-
Table 2. Effect of LH-RH and single treatment of antioestrogen on ovarian and uterine weights in relation to ovulation in rats

|                | Tissue weight (mg) | No. of rats ovulated | No. of ova |
|----------------|-------------------|----------------------|------------|
|                | Ovary             | Uterus               |            |
| Control        | 74.3 ± 2.11       | 283.8 ± 2.08         | 8/8        | 11 ± 0.2 |
| LH-RH          | 75.1 ± 2.30       | 274.0 ± 5.71         | 8/8        | 10 ± 0.8 |
| Tamoxifen      | 61.8 ± 2.32**     | 206.8 ± 6.50***      | 0/4        |         |
| + LH-RH        | 76.8 ± 2.95***    | 216.5 ± 10.31**      | 4/4        | 8 ± 0.8  |
| Clomiphene     | 65.0 ± 2.65*      | 203.5 ± 10.56**      | 2/4        | 9        |
| + LH-RH        | 72.3 ± 3.12       | 203.8 ± 6.42***      | 4/4        | 9 ± 0.5  |
| Tamoxifen      | 61.3 ± 1.70**     | 197.8 ± 6.66***      | 0/4        |         |
| + LH-RH        | 72.5 ± 2.60²      | 216.3 ± 8.28**       | 4/4        | 7 ± 1.9  |
| U-23469        | 64.0 ± 2.04**     | 247.3 ± 6.86**       | 0/4        |         |
| + LH-RH        | 63.8 ± 3.45*      | 190.8 ± 7.65**       | 4/4        | 8 ± 2.0  |
| MPA            | 66.3 ± 1.89*      | 309.3 ± 9.26*        | 0/4        |         |
| + LH-RH        | 65.3 ± 1.18**     | 245.5 ± 13.68**      | 4/4        | 10 ± 0.8 |

Each antioestrogen (1 mg/kg) was administered s.c. at 9:00 a.m. on the first day of diestrus, and LH-RH (500 ng/rat) was administered s.c. at 3:00 p.m. on the day of proestrus. Ovaries and uterus of each rat were isolated at 72 hr after treatment with each antioestrogen. Each value indicates the mean ± S.E. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; ²P < 0.05, ²²P < 0.01 vs. antioestrogen alone.

ments and the ovulation was also inhibited, although clomiphene treatment inhibited them by only 50%. The co-administration of tamoxifen, clomiphene and nafoxidine with LH-RH increased the ovarian weight to control levels and completely reversed the inhibitory effect of these antioestrogens on ovulation. MPA increased the ovarian weight and co-administration of MPA with LH-RH significantly decreased it as compared with the control. Moreover, co-administration of U-23469 with LH-RH produced a fall in the uterine weight as compared with U-23469 alone.

The reduction of 15KD-PGF$_{2\alpha}$, 4BP and menadione reducing activities in the ovarian cytosol was markedly decreased by single administration of each antioestrogen on the first day of diestrus (Fig. 3). 15KD-PGF$_{2\alpha}$ reducing activity was decreased to 45, 65, 50, 50 and 53% of the control levels by treatment with tamoxifen, clomiphene, nafoxidine, U-23469 and MPA, respectively. Furthermore, both 4BP and menadione reducing activities were also decreased significantly to about 30–70% of the control levels by each antioestrogen. These inhibitory effects were completely antagonized by co-administration with LH-RH, although the 15KD-PGF$_{2\alpha}$ reducing activity was not restored by co-administration of MPA with LH-RH and menadione reducing activity was not restored by co-administration of U-23469 or MPA with LH-RH. No significant changes in any of the PNAP and PNBA reducing activities were observed by treatment with each antioestrogen and LH-RH, with the exception of co-administration of clomiphene with LH-RH (Fig. 4).

Significant correlation between 15KD-PGF$_{2\alpha}$ reducing activity and 4BP and menadione reducing activities in the ovarian cytosol of rats was found when antioestrogen and LH-RH were administered.
Fig. 3. Effect of LH-RH and single treatment of antiestrogen on reduction of 15KD-PGF$_2$$\alpha$, 4-benzoylpyrididine and menadione in the ovarian cytosol of rats. Experimental conditions were described in Table 2. Each column indicates the mean ± S.E. □ control, ☐ LH-RH, ☐ antiestrogen alone, ☐ antiestrogen + LH-RH. Tam., tamoxifen; Clo., clomiphene; Naf., nafoxidene; U, U-23469; MPA, medroxyprogesterone acetate. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; # P < 0.05, ##P < 0.01, ###P < 0.001 vs. antiestrogen alone.

Fig. 4. Effect of LH-RH and single treatment of antiestrogen on reduction of 4-nitroacetophenone and 4-nitrobenzaldehyde in the ovarian cytosol of rats. Experimental conditions were as described in Table 2. Each column indicates the mean ± S.E. □ control, ☐ LH-RH, ☐ antiestrogen alone, ☐ antiestrogen + LH-RH. Tam., tamoxifen; Clo., clomiphene; Naf., nafoxidene; U, U-23469; MPA, medroxyprogesterone acetate. *P < 0.05 vs. control; #P < 0.05, ##P < 0.01 vs. antiestrogen alone.

DISCUSSION

It has been well-established that endogenous estradiol can produce a negative as well as positive feed back on gonadotropin release and that the proestrus surge of LH secretion depends on estrogen secretion occurring at diestrus (18). In addition, estrogen receptors have been clearly demonstrated to exist in the medial basal hypothalamus (19) and estrogen has a positive effect on LH-RH synthesis and release in the hypothalamus (20). The aldo-keto reductase family consists of aldehyde re-
ductase (EC 1.1.1.2), aldose reductase (EC 1.1.1.21) and carbonyl reductase (EC 1.1.1.184), a classification based on similar physicochemical properties and substrate specificities (21). In the present study, we investigated the interaction between antiestrogens and LH-RH by determining ovarian aldo-keto reductase activities and ovulation in 4-day cycling rats as a parameter.

Not only was ovulation completely inhibited but also 15KD-PGF$_{2\alpha}$, 4BP and menadione reducing activities in the ovarian cytosol were significantly decreased by both three consecutive treatments and single treatment with each antiestrogen. Ghosh and Nayyar (22) have reported that a single administration of clomiphene on the day of diestrus completely inhibited ovulation in rats, and the inhibitory effect of clomiphene was restored by treatment with hCG. These results suggest that antiestrogen may inhibit the proestrus surge of LH release by inhibiting positive feed back of estrogen in rats. Indeed, we already reported that the inhibition of 15KD-PGF$_{2\alpha}$, 4BP and menadione reducing activities and ovulation by both three consecutive and single administration of antiestrogens was completely antagonized by administration of hCG at 3:00 p.m. on the day of proestrus (23). Namely, it is thought that ovarian aldo-keto reductase, which has 15KD-PGF$_{2\alpha}$, 4BP and menadione reducing activities, may play an important role in the ovulatory process.

However, the effect of MPA on uterine weight, ovulation, and aldo-keto reductase activities appeared to be different from that of other nonsteroidal antiestrogens. Ovulation inhibited by repeated MPA could not be restored after treatment with LH-RH, and single treatment with this antiestrogen significantly increased uterine weight as compared with that of the control. MPA is a progesterone derivative that is different from the other four triphenylethylene derivatives tested here. Kang et al. (24) reported that CI628, an antiestrogen which has progestin action, is a connective tissue mitogen, inducing DNA synthesis in fibroblasts and endothelium. According-ly, these effects in our experiment of MPA seem to be due to progestin action. Furthermore, it is well-known that MPA possesses a glucocorticoid-like action (25). A decrease in adrenal wet weight was observed by three consecutive treatments with MPA but not by other antiestrogen treatment (N. Inaba et al., unpublished data). It is thought that the effect of MPA on ovarian aldo-keto reductase activities towards 15KD-PGF$_{2\alpha}$, 4BP and menadione involves complicated antiestrogenic action including the actions of progesterone and glucocorticoid.

The results that LH-RH treatment on the day of proestrus restored ovulation and 15KD-PGF$_{2\alpha}$, 4BP and menadione reducing activities inhibited by antiestrogen (Figs. 1 and 3, Tables 1 and 2) demonstrate that the inhibitory effect of antiestrogen on the ovary is due to disappearance of the LH surge on the afternoon of proestrus. Gogan et al. (26) indicated that antiestrogen, nitromophene citrate, acted at the pituitary level to decrease its sensitivity to LH-RH rather than direct action on the hypothalamus in female rats. There are many reports that the pituitary is a site for the positive effect of steroids (27, 28). We have also reported that rat ovarian CR activity significantly elevated after the endogenous LH surge during the estrous cycle (15). Therefore, the ovarian enzyme catalyzing the conversion of endogenous 15KD-PGF$_{2\alpha}$ to 13,14H$_2$-PGF$_{2\alpha}$ appeared to be LH dependent; and 13,14H$_2$-PGF$_{2\alpha}$ formed in rat ovary is thought to play an important role in ovarian functions such as ovulation, steroidogenesis and ovum maturation. In fact, we already reported that 13,14H$_2$-PGF$_{2\alpha}$ in vitro stimulated the biosynthesis of progesterone and estradiol from pregnenolone in rat ovary (29).

On the other hand, the enzymes catalyzing predominantly the reduction of PNAP and PNBA in the ovarian cytosol is thought to be not regulated by the hypothalamo-pituitary axis, LH-RH and gonadotropin, since PNAP and PNBA reducing activities were not affected by each antiestrogen and LH-RH. However, the physiological role of these en-
zymes in rat ovary is not clear at present.

Although there is much data about the effects of a number of antiestrogens on the hypothalamus, pituitary and uterus (2, 26, 29–34) obtained from studies determining various endogenous factors, such as LH-RH, PRL, LH and estrogen receptors, the important finding of the present study is that we could assay the pharmacological actions of antiestrogens and LH-RH on gonadotropin release, which is required for ovulation, by determining ovarian aldo-keto reductase activities such as 15KD-PGF$_{2\alpha}$, 4BP and menadione reducing activities.

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