hCG-stimulated ovarian carbonyl reductase in relation to preovulatory ovarian follicular growth

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The relationship between carbonyl reductase, which is localized in the theca and interstitial cells, and ovarian follicular development was evaluated by measuring the enzyme activity and concentration of carbonyl reductase, and examining its immunohistochemical localization in immature rats treated with equine chorionic gonadotrophin (eCG), ovine(o)FSH and hCG. eCG and oFSH were administered s.c. to 26-day-old rats, and the ovaries were isolated 48 and 72 h after treatment. hCG was administered s.c. 48 h (at 28 days of age) after eCG or oFSH, and the ovaries were isolated 6 or 9 h after treatment. Both eCG and oFSH significantly increased the ovarian carbonyl reductase concentration 48 h after treatment compared with the saline-treated group, but the increase with eCG was approximately twice that with oFSH. Seventy-two hours after treatment, eCG further increased both carbonyl reductase activity and its concentration. Ovulation was also induced. oFSH stimulated only ovarian follicular growth without further increase in either activity or concentration. In addition, eCG increased the immunoreactivity to anti-carbonyl reductase antibody in the theca and interstitial cells, but oFSH did not. Treatment with hCG in saline-pretreated rats increased carbonyl reductase activity by 2.8-fold and carbonyl reductase concentration by 4.1-fold after 9 h compared with the 0 h concentration, without producing the large follicles observed with eCG and oFSH. However, the stimulatory effect of hCG on ovarian carbonyl reductase in eCG- or oFSH-pretreated rats was weaker than that in saline-pretreated rats. These results suggest that ovarian carbonyl reductase is induced by LH/hCG but not by FSH and that the enzyme induced by hCG is unrelated to preovulatory ovarian follicular growth.

Introduction

Carbonyl reductase (CR, EC 1.1.1.184) is present in various tissues of mammals, including humans, and catalyses NADPH-dependent reduction of carbonyl compounds, such as 9-ketoprostaglandins and 3-ketosteroids, to the corresponding alcohols (Wermuth, 1985; Maser, 1995). In rats, CR is exclusively expressed in reproductive tissues and the adrenal gland (Iwata *et al.*, 1989, 1990a; Inazu *et al.*, 1994). Ovarian CR activity in rats reaches a maximum on the morning of oestrus after ovulation (Iwata *et al.*, 1990b) and hCG induces the enzyme in oestrogen-pretreated immature rat ovary (Inazu *et al.*, 1990a). Stimulation of both FSH and LH is mandatory according to the two-cell and two-gonadotrophin theory that LH stimulates precursor androgen synthesis in the theca interna cells and FSH stimulates aromatase activity in the granulosa cells. As the ovarian CR is localized in the theca and interstitial cells but not in the granulosa cells (Iwata *et al.*, 1990b), it is thought that the expression of CR is stimulated by LH. However, it is still not clear whether ovarian CR is involved in preovulatory follicular development and the ovulatory process. Chappel and Howles (1991) have reported

that high LH concentration during the preovulatory period may negatively influence postovulatory events. In addition, hCG significantly induces ovarian CR when hCG is administered to anovulatory rats, even though ovulation is not completely restored (Inazu and Satoh, 1994; Inazu and Fujii, 1996). These findings suggest that ovarian CR is not always necessary for follicular maturation and the ovulatory process in the ovary. In the present study, to clarify the relationship between ovarian CR and follicular development during the preovulatory period, changes in the enzyme activity and its concentration, as well as its immunohistochemical localization, were investigated in immature rats after treatment with equine chorionic gonadotrophin (eCG), ovine(o)FSH and hCG.

Materials and Methods

Chemicals

eCG and hCG were obtained from Teikoku Hormone Mfg (Tokyo), and oFSH (NIDDK-oFSH-20) was generously provided by the National Institute of Diabetes and Digestive and

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Fig. 1. Comparison between effects of equine (e)CG and ovine (o)FSH on ovarian carbonyl reductase activity (a) and concentration (b) in immature rats. Rats were given 10 iu eCG, or 2, 5 or 10 μg oFSH s.c. at 26 days of age, and the ovaries of each rat were isolated 0, 48 and 72 h after treatment. Each point shows the mean $\pm$ SEM of four rats. ○, saline; ◇, eCG; ▲, 2 μg oFSH; △, 5 μg oFSH; ▨, 10 μg oFSH. ** $P < 0.01$ versus 48 h after treatment; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus eCG treatment; $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ versus saline treatment.

Kidney Diseases (Bethesda, MD). NADPH was purchased from Oriental Yeast Co. (Tokyo), and 4-benzoylpyridine (4BP) was purchased from Wako Pure Chemical Co. (Osaka). Other chemicals of reagent grade were obtained from Bio-Rad (Tokyo) and Wako Pure Chemical Co.

Animals

Immature female rats (20 days old) of the Wistar-Imamichi strain were purchased from the Institute of Animal Reproduction (Ibaragi) and housed in group cages (four rats per cage) under controlled conditions of light (12 h on, 12 h off) and temperature (24°C). Food and water were always available.

Experimental protocol

Hormone treatment. eCG, hCG and oFSH were each dissolved in 0.9% (w/v) saline solution. eCG (10 iu) and oFSH (2, 5 and 10 μg) were administered s.c. to 26-day-old rats, which were killed 48 and 72 h after treatment, respectively. hCG (10 iu) was administered s.c. to 28-day-old rats, which were killed 6 and 9 h after treatment. To investigate any possible interaction of hCG and eCG or oFSH, 10 iu hCG was administered s.c. to 28-day-old rats which had been treated with eCG (10 iu) or oFSH (2 μg) 48 h before, and rats were killed 6 and 9 h after hCG treatment. Control rats were administered s.c. vehicle alone (0.9% saline solution, w/v) at 26 days of age. The presence of ova in the oviduct was determined under a microscope.

Enzyme assay. The ovaries of each rat were isolated after each gonadotrophin treatment, and homogenized in 4 ml ice-cold phosphate buffer (10 mmol l⁻¹, pH 6.5) containing 1.15% (w/v) KCl, 1 mmol dithiothreitol l⁻¹ and 0.5 mmol EDTA l⁻¹. The homogenate was centrifuged at 9000 g for 20 min at 4°C, and the supernatant obtained was used as source of CR activity. CR activity was measured by using 4BP as a substrate, and the reduction of 4BP (1 mmol l⁻¹) was determined spectrophotometrically in a total volume of 1 ml containing phosphate buffer (0.1 mol l⁻¹, pH 6.5), 0.1 mmol NADPH l⁻¹ and 0.3 ml supernatant. The enzyme activity was expressed as mU mg⁻¹ protein of NADPH oxidized. The protein concentration in the supernatant was determined by the method of Lowry *et al.* (1951), using BSA as a standard.

Determination of enzyme content. The content of ovarian CR in the supernatant was determined by the western blot- peroxidase anti-peroxidase (PAP) method using anti-ovarian CR antiserum raised against the purified rat ovarian CR (Iwata *et al.*, 1989) as described by Inazu *et al.* (1990a). Briefly, 2 μg protein of the supernatant was subjected to SDS-PAGE (10% polyacrylamide gel) and blotted onto polyvinylidene difluoride membrane (Clear Blot Membrane, Atto Co., Tokyo). Enzyme protein on the membrane was visualized by a densitometer (CS-9000, Shimadzu Co., Kyoto) using purified rat ovarian CR as a standard. Two CR isoforms, CR1 and CR2, have been purified previously from rat ovary and two protein bands in the ovarian cytosolic fraction are confirmed by western blot analysis (Iwata *et al.*, 1989). Thus, the protein band which shows a higher molecular mass contains both CR1 and CR2, and the lower band contains a minor form. Furthermore, both bands are induced by eCG and hCG.

Immunohistochemical methods. The ovaries treated with each hormone were isolated quickly, immersed immediately in sodium phosphate buffered (0.1 mol l⁻¹, pH 7.4) 4% (w/v) paraformaldehyde, and fixed for 24 h. After fixation, the samples were rinsed in sodium phosphate buffer (0.1 mol l⁻¹, pH 7.4), sequentially dehydrated in ethanol solution (70–100%, v/v), and embedded in paraffin wax. The paraffin wax blocks were cut into 4 μm thick slices, and immunostaining with anti-rat ovarian CR antiserum was carried out by the

Fig. 2. Western blot analysis of ovarian carbonyl reductase after treatment with equine (e)CG, ovine (o)FSH and hCG in immature rats. Each lane contained 2 µg ovarian cytosolic protein. After SDS-PAGE (10% polyacrylamide gel), western blot analysis was performed using anti-rat ovarian carbonyl reductase antiserum. (a) Time-dependent effects of eCG and oFSH. lane 1, 26-day-old control; lane 2, 28-day-old control; lane 3, 48 h after eCG; lane 4, 48 h after 2 µg oFSH; lane 5, 29-day-old control; lane 6, 72 h after eCG; lane 7, 72 h after 2 µg oFSH. (b) 72 h after eCG and oFSH. lanes 1, 4 and 7, 29-day-old control; lanes 2, 5 and 8, 72 h after eCG; lanes 3, 6 and 9, 72 h after 10 µg oFSH. (c) Effects of hCG and eCG. lanes 1–4, 57 h after saline (28 days of age); lanes 5–8, 9 h after hCG (28 days of age); lanes 9–12, 57 h after eCG (28 days of age).

avidin–biotin peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Lab., CA) as described by Iwata *et al.* (1990b) and Inazu *et al.* (1990b).

Statistical analysis

Experimental data were represented as the mean $\pm$ SEM of four rats. Duncan's multiple range test was used for comparison between groups, with $P < 0.05$ considered to indicate a significant difference.

Results

Effects of eCG and oFSH

eCG increased ovarian mass in a time-dependent manner after treatment, and ovulation was confirmed 72 h later. oFSH did not result in a large increase in ovarian mass compared with that observed in the eCG-treated group, although ovarian mass

was significantly increased by treatment with each dose of oFSH. None of the oFSH doses had any effect on ovulation 72 h later.

Both eCG and oFSH increased the activity and concentration of CR 48 h after treatment compared with saline control (Fig. 1). A marked increase in the enzyme activity and concentration was shown in the eCG-treated ovary 72 h later, while no increase in either enzyme activity or concentration was observed in the oFSH-treated ovary (Figs 1a, b and 2a, b). This was similar to the results in which eCG treatment caused ovulation 72 h after treatment and none of the oFSH doses had any effect on ovulation 72 h later. Significant differences were observed between the effects of eCG on ovarian CR and those of oFSH 72 h after treatment.

Effects of hCG

The time course of effects of hCG on ovarian CR pretreated with eCG and oFSH was investigated (Fig. 3). Both eCG and oFSH were administered to 26-day-old rats 48 h before hCG treatment. In saline-pretreated rats, treatment with hCG caused

Fig. 3. Effects of hCG on ovarian carbonyl reductase activity (a) and concentration (b) in equine (e)CG- or ovine (o)FSH-pretreated immature rats. Rats were given 10 iu eCG or 2 μg oFSH s.c. at 26 days of age, 48 h before hCG treatment. hCG (10 iu) was administered s.c. to 28-day-old rats, and the ovaries of each rat were isolated 0, 6 and 9 h later. Each point shows the mean $\pm$ SEM of four rats. ●, saline pretreatment; ◆, eCG pretreatment; ▲, oFSH pretreatment. * $P < 0.05$, ** $P < 0.01$ versus saline pretreatment; † $P < 0.05$, †† $P < 0.01$ versus 0 h; ‡ $P < 0.01$ versus eCG pretreatment.

2.1- and 2.8-fold increases in CR activity after 6 and 9 h, respectively, compared with at 0 h ($4.29 \text{ mU mg}^{-1} \text{ protein}$) (Fig. 3a). Pretreatment with eCG or oFSH revealed a stimulatory effect of hCG on ovarian CR activity, although ovulation did not occur within 9 h after hCG. hCG increased the enzyme activity to 1.9-fold after 9 h compared with the activity at 0 h in both eCG- and oFSH-pretreated rats. The effect of hCG on CR activity in saline-pretreated rats was greater than that in eCG- and oFSH-pretreated rats. However, time-dependent effects of hCG on the ovarian CR concentration differed from those of CR activity (Fig. 3b). Treatment with hCG in saline-pretreated rats caused the highest increase in the CR concentration; that is, the value ($15.7 \text{ μg mg}^{-1} \text{ protein}$) 9 h after the treatment was 4.1 times that of the 0 h level (Figs 2c

and 3b). In animals pretreated with eCG or oFSH, the stimulatory effect of hCG on CR concentration was weaker than that in saline-pretreated animals. CR concentration 6 h later in eCG- and oFSH-pretreated rats was increased to 1.3 times the 0 h value and, after 9 h, to 1.4- and 1.7 times the 0 h concentration, respectively. Pretreatment with both eCG and oFSH apparently inhibited the stimulatory action of hCG on CR concentration; in particular, in oFSH-pretreated rats, the increase in CR concentration after hCG was significantly inhibited.

Immunohistochemistry

Immunohistochemical localization of ovarian CR after treatment with eCG, oFSH and hCG was investigated using anti-ovarian CR antiserum (Figs 4 and 5). Immunostaining in 28-day-old controls (Fig. 4a) and in 29-day-old controls (Fig. 4b) was weak, and only primary and secondary follicles were present. Treatment with eCG alone (Fig. 4c, e) and oFSH alone (Fig. 4d, f) highly developed secondary to Graafian follicles. Immunohistochemical reactivity in the theca and interstitial cells of the eCG-treated ovary was stronger than that of the ovary treated with oFSH. However, differences between the action of eCG and oFSH were observed in the ovarian sections 72 h after treatment (Fig. 4g, h). Thus, eCG treatment markedly increased the expression of CR, whereas oFSH had no effect on the further expression of CR, even though very large follicles were observed.

Immunohistochemical reactivity in the ovarian cells 9 h after hCG in saline-pretreated rats (Fig. 5a) was stronger than that 57 h after eCG alone (Fig. 4e), although large follicles, such as Graafian follicles, were not found. The ovarian CR concentration 9 h after hCG in saline-pretreated rats was higher than that 57 h after eCG alone (Fig. 2c). In eCG-pretreated rats treated with hCG, the expression of CR in the theca and interstitial cells was increased in agreement with the ovarian follicular growth (Fig. 5b). Similarly, hCG increased the immunoreactivity of ovarian cells in oFSH-pretreated rats (Fig. 5c) compared with that 57 h after oFSH treatment alone (Fig. 4f).

Discussion

Ovarian CR is induced after ovulation in cyclic mature rats (Iwata *et al.*, 1990b) and is stimulated by hCG in immature rats, particularly in ovaries pretreated with oestrogen (Inazu *et al.*, 1990a, b, 1989). From these findings, it is concluded that ovarian CR is LH-dependent. The relationship between the ovarian CR and ovarian follicular growth is explained here in the light of the present results.

The primary signal for beginning preovulatory follicular development is secretion of increased FSH from the pituitary gland. FSH directly stimulates the proliferation of granulosa cells, which are the only cells in females known to possess FSH receptors (Richards *et al.*, 1987). Granulosa, theca and interstitial cells express LH receptors, and LH directly stimulates steroidogenic enzyme systems pre-induced by FSH (Hsueh *et al.*, 1984; Erickson *et al.*, 1985). The secretion of sex steroids by preovulatory follicles and corpora lutea is dependent on LH. LH stimulates the theca-interstitial cells to differentiate and to

Fig. 4. Immunohistochemical localization of ovarian carbonyl reductase after treatment with equine (e)CG and ovine (o)FSH in immature rats. Immunostaining was carried out by the avidin-biotin peroxidase complex method using anti-rat ovarian carbonyl reductase antiserum as a primary antibody. (a) 28-day-old control; (b) 29-day-old control; (c) 48 h after eCG; (d) 48 h after 2 µg oFSH; (e) 57 h after eCG; (f) 57 h after 2 µg oFSH; (g) 72 h after eCG; (h) 72 h after 2 µg oFSH. Scale bar represents 100 µm.

Fig. 5. Immunohistochemical localization of ovarian carbonyl reductase 9 h after treatment with hCG in equine (e)CG- or ovine (o)FSH-pretreated immature rats. Either eCG or oFSH was administered s.c. to 26-day-old rats 48 h before hCG treatment. (a) saline pretreatment; (b) eCG pretreatment; (c) 2 µg oFSH pretreatment. Scale bar represents 100 µm.

secrete androgens. Ovarian CR is localized in these cells and induced by hCG 18 h later with or without ovulation (Inazu *et al.*, 1989, 1990b; Iwata *et al.*, 1990b; Inazu and Satoh, 1994;

Inazu and Fujii, 1996). In the present results, hCG significantly stimulated ovarian CR without ovulation 6 and 9 h after treatment, and the CR was expressed in the theca-interstitial cells stimulated by hCG irrespective of ovarian follicular growth. These results suggest that the hCG-stimulated increase in ovarian CR may be unrelated to the ovulatory process, although the enzyme is dependent on LH action.

It is accepted that eCG possesses both FSH and LH actions. The significant increase in ovarian CR concentration 48 h after eCG, in comparison with that after all doses of oFSH, is thought to be due to LH activity. This suggestion is supported by the results indicating that hCG had a significant stimulatory effect on CR concentration in saline-pretreated rats and that the stimulatory effect of oFSH on CR concentration was very slight. These results suggest that LH may stimulate the expression of ovarian CR before an increase in CR activity in the ovarian cells. Indeed, the increase in CR concentration 6 and 9 h after hCG in saline-pretreated rats was much greater than that in CR activity. Ovarian CR concentration in cyclic mature rats is highest before ovulation, within 9 h after LH surge, and enzyme activity is highest after ovulation, on the morning of oestrus (Iwata *et al.*, 1990b). However, as the enzyme protein concentration induced by hCG is a total of CR isoforms, CR1 and CR2, and immunoreactive protein (which has a lower molecular mass than both CR1 and CR2, to anti-CR antiserum), it is thought that the magnitude of increase in ovarian CR concentration is higher than that in the enzyme activity. The finding that ovulation was caused by eCG 72 h later, but not by oFSH, indicates that both the activities of FSH and LH are needed to stimulate oestrogen synthesis in the granulosa cells and an LH surge, as well as ovarian follicular growth. Accumulation of uterine intraluminal fluid after treatment with oFSH was not as significant as that after treatment with eCG. These results suggest that the action of FSH alone is not sufficient for the expression of ovarian CR and oestrogen synthesis.

In animals pretreated with eCG and oFSH 48 h before hCG treatment, hCG showed similar stimulation of both enzyme activity and concentration, although the values of both of these 48 h after eCG were higher than those after oFSH. This stimulatory effect of hCG was weaker than that of hCG in the case of pretreatment with saline. However, hCG alone did not produce large follicles, such as Graafian follicles and, thus, its action differed from that of eCG and oFSH. Nevertheless, hCG was a stimulatory factor for ovarian CR in rats. These results indicate that it may be LH, rather than FSH, that predominantly regulates ovarian CR in rats, and that the enzyme may not be related to preovulatory follicular growth. The theca-interstitial cells, in which ovarian CR is localized, produce androgens that are precursors for oestrogen synthesis in the granulosa cells (Erickson *et al.*, 1985). However, excessive androgen stimulated by LH has been reported to induce follicle atresia (Erickson *et al.*, 1985). Indeed, it has been demonstrated that, by immunizing against LH or androgens, follicle atresia is inhibited (Scaramuzzi *et al.*, 1977; Terranova and Greenwald, 1981). It is evident that low concentrations of LH are adequate to stimulate the production of androgens during the preovulatory period. As insulin-like growth factor I (IGF-I), inhibin and insulin potentiate the stimulatory effect of LH on androgen synthesis in theca cells (Erickson *et al.*, 1985; Magoffin *et al.*,

1990; Hillier *et al.*, 1991), these hyperstimulations of theca cells may be deleterious to the ovulatory process (Chappel and Howles, 1991). Furthermore, a thecal differentiating factor (TDF) controlled under FSH and secreted by the granulosa cells stimulates androgen production in the theca-interstitial cells independent of LH (Magoffin and Magarelli, 1995; Magarelli *et al.*, 1996) and it has been suggested that abnormal TDF production may lead to hyperandrogenism (Magarelli *et al.*, 1996). Accordingly, one of the physiological roles of ovarian CR induced by LH/hCG may be involved in the process of follicle atresia.

Although it remains unclear whether the stimulatory action of hCG on ovarian CR concentration was inhibited after pretreatment with oFSH, it is thought that an unknown factor(s) produced or activated in the granulosa cells by oFSH may be involved in the inhibition. Epidermal growth factor (EGF) interferes with FSH action, such as expression of LH receptors and increase in aromatase activity (Jones *et al.*, 1982; Knecht and Catt, 1983), and transforming growth factor- β (TGF β) potentiates the response to a low dose of FSH but attenuates the response to a high dose of FSH in cultured rat granulosa cells (Knecht *et al.*, 1987). These findings indicate that ovarian function is regulated by a complex mechanism, such as paracrine and autocrine control, and it is suggested, therefore, that various factors, including gonadotrophins and growth factors may contribute to the regulation of both the activity and concentration of ovarian CR in the theca-interstitial cells.

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