Expression of activities of two 20 α -hydroxysteroid dehydrogenase isozymes in rat corpora lutea

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Summary. The rat ovary contains two isozymes of 20 α -hydroxysteroid dehydrogenase (HSD-1 and HSD-2). In this study, the expression of activity of each isozyme was investigated in ovaries that contained a single generation of corpora lutea during pseudopregnancy. This condition was induced by cervical stimulation in rats that had been rendered anovulatory by housing them in a continuously lit environment. The total activity of cytosolic 20 α -HSD was lower in the ovaries of these pseudopregnant rats than in ovaries containing multiple generations of corpora lutea. In normal pseudopregnancy, HSD-1 activity was low on days 5 and 9 and increased markedly on day 15, whereas HSD-2 was lower than HSD-1 and did not vary throughout pseudopregnancy. However, on days 5 and 9 of continuous-light pseudopregnancy, low activity of HSD-1 only was detected; by day 15, HSD-1 activity had increased sixfold and HSD-2 activity could be detected.

Immunohistochemical methods using a specific antibody recognizing both HSD-1 and HSD-2 revealed that the number of 20 α -HSD-positive luteal cells increased by day 15. Thus, the increase in total enzyme activity and appearance of HSD-2 activity observed at late pseudopregnancy was accompanied by an increase in the number of 20 α -HSD-positive luteal cells.

Keywords: 20 α -hydroxysteroid dehydrogenase; pseudopregnancy; corpus luteum; rat

Introduction

The enzyme 20 α -hydroxysteroid dehydrogenase (20 α -HSD) was first isolated from rat ovaries (Wiest, 1959; Wiest & Wilcox, 1961) and plays a key role in converting progesterone to a biologically inactive steroid, 20 α -hydroxypregn-4-en-3-one (20 α -dihydroprogesterone). The activity of 20 α -HSD is associated with luteal regression (Wiest *et al.*, 1968) and is known to increase at the termination of pseudopregnancy and pregnancy (Kuhn & Briley, 1970). Cytosolic 20 α -HSD is derived from both luteal and non-luteal ovarian tissue, activity in the former being 3–5 times higher than that in the latter at the end of pseudopregnancy (Matsuda *et al.*, 1990).

We have purified 20 α -HSD and found two distinct 20 α -HSD molecules designated HSD-1 and HSD-2, which have different electric charges, Michaelis constants and substrate specificities (Noda *et al.*, 1991). The expression of these activities differed during the course of pseudopregnancy: the ovarian HSD-1 concentration was constant whereas the concentration of HSD-2 gradually decreased with the advance of pseudopregnancy (Shiota *et al.*, 1988). These data were obtained using rat ovaries that carried multiple generations of corpora lutea, and thus further study was needed to determine which component of ovarian tissue is responsible for this change in enzymatic

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activities. In this study, the expression of each type of 20 α -HSD was investigated in pseudopregnant rats possessing a single generation of corpora lutea.

Materials and Methods

Reagents

DEAE Toyopearl 650S was obtained from Toso Co. Ltd, Tokyo, Japan. Nicotinamide adenine dinucleotide phosphate (NADP) and 20 α -dihydroprogesterone were purchased from the Sigma Chemical Co., St Louis, MO, USA. The protein assay reagent was obtained from Bio-Rad Laboratories, Richmond, CA, USA. Phenylmethanesulphonyl fluoride, leupeptin and all other chemicals were purchased from Wako Pure Chemicals (Japan) and all were of analytical grade.

Animals

Female Wistar–Imamichi rats (body weight 200–250 g, 10–12 weeks of age) were housed under controlled lighting conditions of 14 h light:10 h dark (lights on 05:00–19:00 h; normal condition) and at a temperature of 22.0°C. Vaginal smears were taken daily. Only animals that had shown regular 4-day oestrous cycles for more than two cycles were used. Some animals were rendered pseudopregnant by cervical stimulation applied between 18:00 and 19:00 h on the pro-oestrous day. Some other regularly cycling rats under a normal light regimen were transferred to an isolated room in which continuous lighting was supplied. Animals that had shown continual vaginal epithelium cornification for more than 15 days under continuous lighting were used, and ovulation and subsequent pseudopregnancy was induced by cervical stimulation between 18:00 and 19:00 h (Takahashi *et al.*, 1977). The day after stimulation were designated day 0 of pseudopregnancy. Rats induced to be pseudopregnant in continuous light were kept under continuous light until they were killed.

Tissue sampling and collection of blood

Pseudopregnant animals were killed by decapitation between 11:00 and 13:00 h on days 5, 9 and 15 of pseudopregnancy. Cycling rats at dioestrus (day after metoestrus) were killed between 18:00 and 20:00 h. The ovaries from pseudopregnant rats were removed, freed from adherent tissues and stored at -80°C until use. Of the ovaries from dioestrous rats, corpora lutea were identified macroscopically and as many as possible were enucleated. Thus, the ovarian tissue from dioestrous rats was separated into either luteal tissue or non-luteal tissue.

Blood samples were collected throughout the period of pseudopregnancy, allowed to clot at room temperature for 2 h and centrifuged at 1500 *g* for 15 min. The sera thus obtained were stored at -20°C until the hormone assay.

Preparation of rat ovarian cytosol

Each frozen ovarian tissue was weighed and homogenized in 20 volumes of potassium phosphate buffer (5 mmol l^{-1} , pH 7.0) containing 1 mmol EDTA l^{-1} , 1 mmol dithiothreitol l^{-1} and 10% glycerol. Protease inhibitors, 1 mmol phenylmethanesulphonyl fluoride l^{-1} and 50 mg leupeptin ml^{-1} , were added immediately before homogenization. The homogenate was centrifuged at 105 000 *g* for 90 min. The final supernatant was used as the cytosol fraction. All procedures were performed at 4°C.

Measurement of activity of 20 α -hydroxysteroid dehydrogenase

Activity of 20 α -HSD was measured by the method of Wiest *et al.* (1968) with a few modifications. The assay medium was Tris-HCl buffer solution (0.1 mol l^{-1} , pH 8.0) containing 30 μmol 20 α -dihydroprogesterone l^{-1} , 300 μmol NADP l^{-1} , 1 mmol EDTA l^{-1} , 5 mmol dithiothreitol l^{-1} , and 3% ethanol for steroid solubilization; dithiothreitol and NADP were added immediately before use; 500 μl of the assay medium was warmed to 37°C in a special microcuvette (0.7 ml). The enzyme reaction was initiated by introducing a 25 μl sample into the cuvette with rapid mixing. The temperature during the assay was maintained at 37°C. Initial velocities were followed spectrophotometrically at 340 nm for 3 min with a Hitachi U-2000 Spectrophotometer (a molar absorbance of 6220 at 340 nm was used to calculate NADPH concentrations). One unit of enzyme activity was defined as the amount that could reduce 1 μmol NADP min^{-1} at 37°C.

Separation of HSD-1 and HSD-2

The ovarian cytosol sample was applied to a DEAE-Toyopearl (0.7 $\times$ 6.7 cm, 650S) anion-exchange column on a high-performance liquid chromatography system (Waters model 650: Waters, Bedford, MA). Chromatography was

performed at 1 ml min⁻¹ using 5 mmol potassium phosphate buffer solution l⁻¹ (pH 7.0) containing 1 mmol EDTA l⁻¹, 1 mmol dithiothreitol l⁻¹ and 10% glycerol (buffer A) and eluted with a linear gradient of increasing KCl concentration in buffer A. Eluates were fractionated into 500 μ l per tube and the enzyme activity was measured as already described. The HSD-1 eluted between fractions 13 and 17, and HSD-2 between fractions 21 and 22 (Noda *et al.*, 1991).

Steroid hormone assays

Concentration of serum 20 α -dihydroprogesterone was determined by radioimmunoassay using specific antibodies raised in our laboratory as described by Matsuyama *et al.* (1990).

Histological studies by indirect immunofluorescence

The antibody against 20 α -HSD was generated in 6-week-old ICR mice by repeated intradermal injections of the purified HSD-2 preparation emulsified in complete Freund's adjuvant. Serum was collected and purified into an IgG fraction by protein A Sepharose chromatography. The reactivity of the antibody was examined against the purified preparations of HSD-1 and HSD-2 by the enzyme-linked immunosorbent assay, using anti-mouse IgG labelled with horseradish peroxide *o*-phenylenediamine. At the dilution of 1:100 or 1:1000 of the antiserum, the absorbances at 490 nm were 0.43 or 0.06 for HSD-1 and 0.45 or 0.055 for HSD-2, respectively, indicating that this antibody showed almost equal reactivity towards HSD-1 and -2, probably because of the similarity in the amino acid sequence of these isozymes.

Indirect immunofluorescence histology was performed on ovarian sections from continuous-light pseudopregnant rats. Sections, 5 μ m thick, were prepared from specimens frozen in liquid nitrogen.

The sections were first exposed to the antibody at 1:80 dilution for 20 h at 4°C and then rinsed with phosphate buffer solution and incubated for 4 h at room temperature with fluorescein-conjugated rabbit anti-mouse IgG (MBS, Japan) (1:30, v/v). After further rinsing with phosphate buffer solution, they were mounted in 50% glycerol in phosphate buffer solution. The specimens were then observed with a Nikon fluorescence microscope.

Protein assay

Protein was quantified by the method of Bradford (1976) using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard.

Statistical analysis

Duncan's multiple-range test was used for statistical evaluation of the results. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Activity of 20 α -HSD in luteal and non-luteal tissue

In cycling rats on the day of dioestrus, activities of both isozymes of 20 α -HSD were detected in luteal and non-luteal tissue (Table 1), but the activity of each was much higher in luteal than in non-luteal tissue. The total activities of 20 α -HSD in the unfractionated and fractionated samples were almost the same, suggesting removal of some unknown inhibitor during fractionation.

Activity of 20 α -HSD in the cytosol of ovarian cells of pseudopregnant rats

The 20 α -HSD activity (the samples were not fractionated into HSD-1 and -2) of cytosol of ovarian cells was assayed on day 5, 9 or 15 (Fig. 1). In normal pseudopregnant rats, the activity was high on days 5 and 9 and increased further on day 15. In pseudopregnant rats in continuous light, activities on days 5 and 9 were one-sixth of those in normal rats. Although the activity increased on day 15, it was only half that of normal rats. Concentrations of 20 α -dihydroprogesterone from day 4 to 12 were similar in normal and continuous-light rats, being highest on days 14 and 15 (Fig. 2).

Table 1. 20 α -hydroxysteroid dehydrogenase (20 α -HSD) activities of the luteal and non-luteal tissue from the ovary of cycling rats on the day of dioestrus before and after DEAE-Toyopearl anion exchange chromatography

Tissue	20 α -HSD activity			
	Unfractionated	Fractionated		Total
		HSD-1	HSD-2	
Luteal	102.1 $\pm$ 19.8*	53.5 $\pm$ 28.6	54.2 $\pm$ 9.9	108.4 $\pm$ 15.5
Non-luteal	20.5 $\pm$ 1.9*	11.9 $\pm$ 0.7*	6.3 $\pm$ 1.3*	18.1 $\pm$ 2.5*

20 α -HSD activity is expressed as nmol NADPH min⁻¹ per pair of ovaries.
*Each value represents the mean $\pm$ SEM for six animals.
*Significantly different from corresponding luteal tissue ($P < 0.05$).

Fig. 1. Activity of ovarian cytosolic 20 α -hydroxysteroid dehydrogenase (20 α -HSD) in pseudo-pregnant rats in (a) normal or (b) continuous light conditions. Each column and vertical bar represents the mean $\pm$ SEM for 3–5 animals. The enzyme activity was expressed as mmol NADPH min⁻¹ ml⁻¹, with 1 unit of activity defined as the amount that can induce 1 mmol NADPH min⁻¹ ml⁻¹ at 37°C. *Significantly different from corresponding values at days 5 and 9 of pseudopregnancy ($P < 0.05$).

Fig. 2. Concentration of 20 α -dihydroprogesterone in serum of pseudopregnant rats in normal (○) or continuous (●) light conditions. The day after cervical stimulation was designated day 0 of pseudopregnancy. Each column and vertical bar represents the mean $\pm$ SEM for 3–5 animals.

Fig. 3. High-performance liquid chromatography profiles of activities of 20 α -hydroxysteroid dehydrogenase (●) and protein concentration (○) in ovaries of rats on a preparative DEAE-Toyopearl anion-exchange column (0.7×6.7 cm², 650S) using (a) day 9 and (b) day 15 pseudopregnant rats in continuous light conditions. Chromatography was performed at 1 ml min⁻¹ with a linear gradient of KCl (—): for 0–50% 0.5 mol KCl l⁻¹ for 10 min, 50–100% for 5 min and 100% of 0.5 mol KCl l⁻¹ for 5 min. Activity of 20 α -hydroxysteroid dehydrogenase was assayed using 20 α -dihydroprogesterone as a substrate.

On day 9 in rats in continuous light only HSD-1 activity was observed (Fig. 3a) but, on day 15 (Fig. 3b), HSD-2 activity was detected together with an increase in HSD-1 activity. In normal rats, activity of HSD-1 increased towards the end of pseudopregnancy and activity of HSD-2 decreased, in accordance with a previous report (Noda *et al.*, 1992). In rats exposed to continuous light, activity of HSD-1 also increased towards the end of pseudopregnancy although values were lower than those in normal rats, and HSD-2 was detected only on day 15 (Fig. 4).

Distribution of anti-20 α -HSD-positive cells in the corpus luteum

Immunoreactive 20 α -HSD was localized in the luteal cells from pseudopregnant rats in continuous light conditions on day 9 (Fig. 5a). Within one corpus luteum, luteal cells staining positively and negatively with anti-20 α -HSD were observed. Both types of cell, with an increased proportion of those staining positively, were found in pseudopregnant rats in continuous-light conditions on day 15, but the follicle was not stained (Fig. 5b).

Fig. 4. Changes in activities of 20 α -hydroxysteroid dehydrogenase-1 (□) and 20 α -hydroxysteroid dehydrogenase-2 (■) in ovaries of pseudopregnant rats in (a) rats in normal light conditions or (b) rats in conditions of continuous light. Activities were measured after ovarian cytosolic samples had been subjected to DEAE-Toyopearl anion-exchange chromatography on high-performance liquid chromatography. ND: not detected. Each column and vertical bar represents the mean $\pm$ SEM for 3–5 animals. Within a measurement, means with different superscripts differ significantly ($P < 0.05$).

Discussion

The activities of HSD-1 and HSD-2 were investigated by biochemical and immunohistochemical methods in the ovary of pseudopregnant rats kept in conditions of continuous light. The ovary of pseudopregnant rats kept in continuous light carries only a single generation of corpora lutea (Takahashi & Suzuki 1969; Murakami *et al.*, 1982). We confirmed our previous results that in normal pseudopregnant rats, 20 α -HSD-2 activity decreased and 20 α -HSD-1 activity increased in late pseudopregnancy (Noda *et al.*, 1992). In a similar way to normal rats, HSD-1 activity was dominant in rats exposed to continuous light throughout the period of pseudopregnancy. The activity of 20 α -HSD-2 was detected only at late pseudopregnancy. Changes in activity of each isozyme during the course of pseudopregnancy therefore differed between normal rats and rats in continuous-light conditions. This difference is due to the different cellular components of these ovaries: the ovary from normal pseudopregnant rats contains multiple generations of corpora lutea, whereas that of continuous-light pseudopregnant rats contains a single generation. These results therefore indicate that (i) corpora lutea of older generations contribute more to whole ovarian 20 α -HSD activity, (ii) newly formed corpora lutea contain only 20 α -HSD-1 activity, and (iii) old corpora lutea express activities of each HSD isozyme as shown in the luteal tissue of cycling rats on the day of dioestrus where only degenerating old corpora lutea exist.

Ovarian 20 α -HSD has been studied histologically in normally cycling rats using the substrate–enzyme reaction (Wiest *et al.*, 1963, 1968; Pupkin *et al.*, 1966). Enzyme activity was detected in fresh corpora lutea in the afternoon of the day of metoestrus and more intensely on the next day (dioestrus). We demonstrated the presence of immunoreactive 20 α -HSD in luteal cells on day 9 of pseudopregnancy and the reactivity increased towards the end of pseudopregnancy with a concomitant increase in the number of 20 α -HSD-positive cells. The increase in 20 α -HSD activity identified in this study seems to be related to an increase in the number of 20 α -HSD-positive cells. The 20 α -dihydroprogesterone levels after day 4 of pseudopregnancy were virtually identical in normal rats and in rats kept in continuous light, although the total activity of 20 α -HSD in the whole ovary of pseudopregnant rats kept in continuous light was less than half that of normal pseudopregnant rats. The main site of metabolism of progesterone to 20 α -dihydroprogesterone must be the luteal cells of newly formed corpora lutea. Where there is 20 α -HSD activity in ovarian tissue other than

Fig. 5. Sections (5 μ m thick) of ovarian tissue from a normal pseudopregnant rat on (a) day 9 ($\times 189$) and (b) day 15 ($\times 95$). The immunoreactivity with anti-20 α -hydroxysteroid dehydrogenase antibody is demonstrated by the indirect immunofluorescence. Note granular fluorescence (arrows) in the cytoplasm of luteal cells (a) and intensely stained luteal cells (b). The follicle (arrow) was not stained (b).

the corpus luteum, a much lower contribution to progesterone catabolism would be made owing to a lower local concentration of the substrate (progesterone) than in luteal cells where progesterone is synthesized. Furthermore, HSD-1 was found to be the major subtype responsible for progesterone catabolism. Activity of HSD-2 may not be involved in the luteolytic process at all, since its activity was minimal even at day 15 of pseudopregnancy.

We previously found that the enzymatic activity increased when the sample was passed through a DEAE column (Noda *et al.*, 1991), which suggests that there is a 20 α -HSD-regulatory substance which alters enzymatic activity. In the present study, a slight increase was seen after DEAE chromatography in ovarian cytosolic preparations from dioestrous rats. The role of 20 α -HSD

activity in the residual tissue of the ovary or aged corpora lutea cannot be wholly explained by the discussion above. We previously found multiple types of 20 α -HSD in spontaneous neoplasms and suggested a possible role for a survival factor in tumour cells, which protects them from the cytotoxic action of progesterone by metabolizing it to the inactive hormone 20 α -dihydroprogesterone (Shiota *et al.*, 1991).

Progesterone is known to be cytotoxic to several types of cell (Fuks & Weinstein, 1979). During normal oestrous cycles, corpora lutea begin to express 20 α -HSD activity 3.5 days after ovulation and thus activity is maintained for at least 9 days when luteal cells are phagocytosed by macrophages (Pupkin *et al.*, 1966). The activity of 20 α -HSD may be related to a survival mechanism of those luteal cells remaining in the ovary.

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