Inhibition of ovulation, steroidogenesis and collagenolytic activity in rabbits by sulpiride-induced hyperprolactinaemia*

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Summary. Ovulation induced by hCG in rabbits was reduced significantly ($P < 0.005$) by sulpiride-induced hyperprolactinaemia. The pre- and post-ovulatory increases in peripheral and ovarian venous progesterone (but not oestradiol or testosterone) were suppressed in the treated animals. The condition of hyperprolactinaemia also prevented the usual changes in 2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH peptidase (DNP-peptidase) and α -N-benzoyl-DL-Arg- β -naphthylamide hydrolase (BANA-hydrolase) activities in follicular tissue that had been stimulated by an ovulatory dose of hCG. These results suggest that inhibition of progesterone production and collagenolytic enzyme activity by sulpiride-induced hyperprolactinaemia may be responsible for the ovulatory dysfunction that occurs when a mammal has a high level of circulating prolactin.

Keywords: ovulation; steroidogenesis; proteolytic enzyme; hyperprolactinaemia; rabbit

Introduction

Sulpiride has been used extensively to induce hyperprolactinaemia and to study how this condition affects the menstrual cycle (L'Hermite *et al.*, 1978a; Aso *et al.*, 1982). However, it is not yet clear whether the abnormal elevation in circulating prolactin has a direct effect on ovulation, the principal event of the menstrual cycle which occurs as a consequence of a number of complex biochemical events in the ovary (Espey, 1980). Ovarian steroidogenesis has been demonstrated to be one of the important events leading to ovulation by the observation that the administration of inhibitors of steroidogenesis could block ovulation in rats (Lipner & Greep, 1971). The connective tissue of the ovarian follicle undergoes deterioration and this change is associated with dissociation of the follicular collagen before ovulation (Espey, 1967; Okamura *et al.*, 1980). Collagenolytic enzyme activity in the ovary is observed with α -N-benzoyl-DL-Arg- β -naphthylamide (BANA) as the substrate, and this activity changes in relation to ovulation (Fukumoto *et al.*, 1981; Kawamura *et al.*, 1984). Moreover, collagen-analogous synthetic substrate, 2,4-dinitrophenyl(DNP)-Pro-Leu-Gly-Ile-Ala-Gln-D-Arg peptide, digestible activity is observed in the Graafian follicle (Morales *et al.*, 1978; Okamura *et al.*, 1985). In an effort to clarify the mechanism by which prolactin interferes with these chemical events of ovulation, we have injected sulpiride-treated hyperprolactinaemic rabbits with an ovulatory dose of hCG and then measured (a) peripheral and ovarian venous concentrations of oestradiol, progesterone and testosterone, and (b) the ovarian tissue levels of DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH peptidase (DNP-peptidase) and BANA-hydrolase.

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Materials and Methods

Animals. Virgin Japanese White rabbits were used. These 48 sexually mature animals weighed 2.8–3.5 kg. They were housed in air-conditioned quarters with a daily light cycle of 14 h and fed pressed food and water *ad libitum* for a minimum of 3 weeks before use.

Induction of hyperprolactinaemia. Hyperprolactinaemia was induced by injecting sulpiride (Fujisawa Pharmaceutical Co. Ltd, Tokyo, Japan) into the subcutaneous tissue of the neck at a dose of 8 mg/kg 3 times daily (at 09:00, 14:00 and 19:00 h) for 4 days. Control animals were injected with saline (0.9% w/v) only.

Blood sample collection. Peripheral blood samples were taken with the heparinized syringe from the marginal ear vein. Ovarian venous blood was withdrawn through a 25-gauge hypodermic needle after the rabbits were anaesthetized with pentobarbitone sodium and a laparotomy was performed. All samples were stored at -20°C until assayed for the specified hormones.

Ovulation induction and confirmation. At 20:00 h on the 4th day of sulpiride treatment, the experimental animals were injected in the marginal ear vein with 100 i.u. hCG (Mochida Pharmaceutical Co. Ltd, Tokyo, Japan). Ovulation was confirmed 14 h later by observing the number of ruptured follicles or fresh corpora lutea on each ovary under a dissecting microscope.

Follicle collection and extraction. Whole ovaries were placed on a chilled plate under a dissecting microscope. The mature follicles (>1 mm in diameter) were cut free of the stromal connective tissue and proportionally distributed into two groups for the assay of DNP-peptidase and BANA-hydrolase activity, respectively. They were frozen at -70°C until assays were performed.

Prolactin assay. Plasma prolactin concentrations were determined by a specific homologous double-antibody radioimmunoassay kit (National Hormone and Pituitary Program, Baltimore, MD, U.S.A.). Highly purified rabbit prolactin (NIADDK-AFP-1974-C) was used for the standard curve and for radioiodination, which was performed by a modified lactoperoxidase procedure (Frantz & Turkington, 1972; Harigaya *et al.*, 1982). Initially, the assay tubes contained 100 μl diluted rabbit plasma (or prolactin standard), 100 μl 0.05 M-phosphate-buffered saline (PBS, containing 1% bovine serum albumin, and 2.7 mM-EDTA), and 100 μl of rat anti-rabbit prolactin serum (NIADDK-AFP-18102677, diluted $\times 200\,000$ with PBS containing 2% normal rat serum). These tubes were incubated at 4°C for 48 h and then 100 μl ^{125}I -labelled prolactin (containing 15 000–20 000 c.p.m.) were added to each tube and they were incubated for another 24 h. Finally, 200 μl of a 1:20 dilution of goat anti-rat gamma globulin (Cappel Laboratories, Cochranville, PA, U.S.A.) were added to the assay tubes and they were incubated for another 48 h. At the end of this incubation, the tubes were centrifuged at 2000 *g* for 30 min to separate the bound and free ^{125}I -labelled prolactin. The radioactivity in the pellet was counted after aspirating the supernatant. The assay sensitivity was 0.39 ng/ml. The intra- and interassay coefficients of variation were 8.9% and 13.5%, respectively, for the duplicate samples.

Gonadotrophin assays. Plasma LH and FSH concentrations were measured by specific double-antibody radioimmunoassay kits (National Hormone and Pituitary Program). Highly purified rabbit LH (NIADDK-AFP-559-3) and FSH (NIADDK-AFP-538-C) were used for the standard curves and for radioiodination, which was performed by a lactoperoxidase method (Miyachi *et al.*, 1972). Guinea-pig antisera against rabbit LH (NIADDK-AFP-81-28) and FSH (NIADDK-AFP-4-7-21-76) were supplied with the kits. Initially, the assay tubes contained 100 μl rabbit plasma (or 100 μl LH or FSH standard), 100 μl 0.05 M-PBS and 100 μl of the anti-rabbit LH or FSH (diluted $\times 900\,000$ and $\times 52\,000$, respectively, with PBS). These tubes were incubated at 4°C for 24 h and then 100 μl ^{125}I -labelled LH or FSH (containing 15 000–20 000 c.p.m.) was added and the tubes were incubated for another 24 h. Subsequently, 200 μl of a 1:20 dilution of goat anti-guinea-pig gamma globulin (Cappel Laboratories) were added to the assay tubes and they were incubated for an additional 84–96 h. At the end of this incubation, the tubes were centrifuged at 2000 *g* for 30 min to separate the bound and free ^{125}I -labelled LH or FSH. The radioactivity in the pellet was counted after aspirating the supernatant. The sensitivity of the LH assay was 0.63 ng/ml and that of the FSH assay was 0.78 ng/ml.

Steroid assays. Plasma steroids were determined by radioimmunoassay as described previously (Mori *et al.*, 1980). Antisera against oestradiol, progesterone and testosterone were purchased from Teikoku Hormone Mfg. Co. Ltd, Tokyo, Japan. The assay sensitivities were 10 pg oestradiol/ml, 25 pg progesterone/ml and 10 pg testosterone/ml plasma, respectively. The intra- and interassay coefficients of variation for all steroids were $<10\%$ and $<15\%$, respectively.

DNP-peptidase activity assay. The enzyme activity was assayed by the method of Morales *et al.* (1978) as modified by Fukumoto *et al.* (1981) and Kawamura *et al.* (1984). The follicular tissues were washed with 50 mM-Tris-HCl buffer (pH 7.6) containing 0.15 M-NaCl and 5 mM- CaCl_2 (assay buffer) and homogenized in the assay buffer with a glass homogenizer and a Teflon homogenizer to a concentration of 30 mg tissue/ml. The homogenates were incubated at 42°C for 30 min for heat shock and cooled, then centrifuged at 20 000 *g* for 10 min. The supernatants were passed through the 45 μm Millipore filter (Millipore Corp., Bedford, MA, U.S.A.) and the filtrates were used as the enzyme solution. The synthetic DNP-peptide (Peptide Institute, Protein Research Foundation, Osaka, Japan) was dissolved in assay buffer and centrifuged at 10 000 *g* for 10 min and the supernatant was used as the substrate solution. The

reaction mixture, which consisted of 150 μ l substrate, 50 μ l enzyme solution and 100 μ l assay buffer, was incubated at 37°C for 3 h. The reaction was terminated with 300 μ l 1.0 N-HCl and the cleaved peptide was extracted with 1.5 ml ethyl acetate and quantitated by absorbance (A) at 365 nm. The activity was expressed as Δ optical density (OD) after the absorbance value of a 0 time incubation blank was subtracted from those of an equivalent tube that had been incubated for 3 h.

BANA-hydrolase activity assay. The method of Barrett (1972), as confirmed by Fukumoto *et al.* (1981) and Kawamura *et al.* (1984) was used. The follicular tissues were washed with saline (0.9% (w/v) NaCl) and homogenized in 0.25 M-sucrose containing 0.1% Triton-X 100 to a concentration of 30 mg tissue/ml. The homogenates were centrifuged at 20 000 g for 10 min and the supernatants were used as the enzyme solution. The reaction mixture, which consisted of 50 μ l enzyme solution, 15 μ l BANA substrate (Sigma, Chemical Co., St Louis, MO, U.S.A.) and 450 μ l assay buffer (0.1 M-phosphate buffer in 1 mM-EDTA, pH 6.0), was incubated at 37°C for 10 min. The reaction was stopped by the addition of 600 μ l of coupling reagent, which contained 300 μ l *p*-chloromercuribenzoate (Sigma) in EDTA-PBS and 300 μ l 6% Fast Garnet GBC (Sigma) and the released β -naphthylamide was extracted with 1.2 ml of butanol and determined by measuring the absorbance of the organic layer at 520 nm.

Statistical analysis. The results are expressed as means $\pm$ s.e.m. Significant differences between the groups were determined by unpaired Student's *t* tests, except for the values of ovulation rate which were analysed by χ^2 tests.

Results

Effects of sulpiride on plasma prolactin and ovulation

Plasma prolactin concentration increased 11-fold during the 4 days of sulpiride treatment (Fig. 1), and was still significantly elevated at 14 h after hCG administration. The ovulation rate at 14 h after hCG was significantly lower ($P < 0.005$) in sulpiride-treated (61.5%) than in control rabbits (84.1%). (The amounts of LH and FSH in all samples were below the sensitivity levels of the respective radioimmunoassays.)

Fig. 1. Plasma prolactin concentrations in the control (○) and sulpiride-treated (●) animals. Transverse hatched bar indicates the period of sulpiride treatment and arrow denotes the time of hCG injection. There were 6 rabbits per group. * $P < 0.005$, ** $P < 0.001$ compared with the corresponding control values.

Effect of hyperprolactinaemia on steroidogenesis

There were no significant differences in the peripheral plasma concentrations of oestradiol, progesterone, and testosterone between the control and the sulpiride-treated rabbits during the 4

Fig. 2. Oestradiol, progesterone and testosterone concentrations in peripheral plasma (a, b, c) and ovarian (d) venous plasma of the control (○ and open bars) and sulpiride-treated (● and solid bars) groups. Ovarian venous plasma was taken at 14 h after hCG (▲). Transverse hatched bars indicate the period of treatment and arrows denote the time of hCG injection. There were 6 rabbits per group. * $P < 0.05$, ** $P < 0.005$ compared with the corresponding control values.

days of treatment (Fig. 2). However, at 14 h after hCG, peripheral progesterone (but not oestradiol or testosterone) concentrations were substantially increased, but the increase was significantly less in the sulpiride-treated animals (Fig. 2b). This difference in the progesterone values between the control and sulpiride-treated rabbit was also detected in the ovarian venous plasma at 14 h after hCG (Fig. 2d). The similar reduction in the peripheral progesterone concentrations in the sulpiride-treated group could be detected as early as 1–4 h after hCG administration (Fig. 3b), but no differences were found in the concentrations of oestradiol and testosterone (Figs 3a, c) during the same period after hCG. Also, the elevation in the ovarian venous concentration of progesterone at 4 h after hCG was significantly suppressed in the sulpiride-treated group as compared with the control group (Fig. 3d).

Effect of hyperprolactinaemia on peptidase activities

In the control animals, activities of DNP-peptidase and BANA-hydrolase were higher at 8 h after hCG than at 10 h after hCG when follicular rupture is more imminent (Fig. 4). In contrast, in sulpiride-treated animals, the activities of both enzymes remained elevated at 10 h after hCG, and were significantly higher than in the respective controls.

Discussion

This study shows that sulpiride-induced hyperprolactinaemia suppresses not only the steroidogenesis that is characteristic of ovulation, but also the usual changes in ovarian proteolytic enzymes.

Fig. 3. Oestradiol, progesterone and testosterone in peripheral plasma (a, b, c) and ovarian (d) venous plasma of the control (○ and open bars) and sulpiride-treated (● and solid bars) groups. Ovarian venous plasma was taken at 4 h after hCG. Transverse hatched bars indicate the period of sulpiride treatment and arrows denote the time of hCG injection. There were 6 rabbits per group. * $P < 0.005$, ** $P < 0.001$ compared with the corresponding control values.

Fig. 4. DNP-peptidase (a) and BANA-hydrolase (b) enzyme activities at 8 and 10 h after hCG in the follicular tissues of the control (open bars) and sulpiride-treated (solid bars) rabbits. There were 3 rabbits per group per enzyme at 8 h and 10 h after hCG, respectively. * $P < 0.01$, ** $P < 0.001$ compared with values of the control group.

Sulpiride is a dopamine antagonist which acts on the lactotrophes of the adenohypophysis to induce prolactin secretion (MacLeod & Robyn, 1977), and its disruption of normal events of the menstrual cycle could be due either to some direct effect of sulpiride on endocrine mechanisms that regulate the menstrual cycle and ovulation, or to the hyperprolactinaemia that is brought about by the sulpiride treatment (L'Hermite *et al.*, 1978b). However, the former does not seem likely since

there are reports that this agent does not affect the tonic secretions of other pituitary hormones even if it is administered chronically throughout the follicular phase of a cycle (Robyn *et al.*, 1977; L'Hermite *et al.*, 1978b). Although we do not have direct evidence to confirm this report (because the LH and FSH concentrations were too low to detect), our observation that there was no significant difference in the oestradiol concentrations between the treated and untreated groups suggests that there was adequate gonadotrophin secretion for normal follicular maturation. After an ovulatory dose of hCG injection we observed a reduction in the normal pre- and postovulatory production of progesterone in the sulpiride-treated animals, demonstrating that the elevated concentration of prolactin might act directly on the granulosa cells to desensitize them to gonadotrophin stimulation. This possible direct effect at the ovarian level is supported by the results of in-vitro studies of the granulosa (McNatty *et al.*, 1974; Demura *et al.*, 1982). Hamada *et al.* (1980) also indicated that at certain high levels, prolactin, independent of hCG, interferes with follicular rupture by the observation using a preparation of the in-vitro perfused rabbit ovary.

An abrupt preovulatory rise in progesterone has been observed about 12 h before the mid-cycle LH surge in humans (Hoff *et al.*, 1983) and within 5 h after mating or hCG administration in rabbits (Hilliard & Eaton, 1971; Mills & Savard, 1973; Wu *et al.*, 1977). The idea that progesterone may be important in ovulation is supported by evidence that hCG-induced ovulation can be blocked by treating rats with inhibitors of 3β -hydroxysteroid dehydrogenase which interrupt the steroidogenic pathway to progesterone (Lipner & Greep, 1971) and this blockage can be overcome by exogenous progesterone (Snyder *et al.*, 1984). Furthermore, anti-progesterone antiserum inhibits ovulation in rats (Mori *et al.*, 1977a), and a dose of cycloheximide that inhibits ovulation in rabbits also inhibits the normal steroidogenic activity that precedes ovulation (Espey, 1986). Collectively, these observations suggest that progesterone may be a prerequisite to ovulation. If it is true, then the inhibition of ovulation by sulpiride treatment may be a result of the decline in progesterone secretion (by granulosa cells) under the conditions of hyperprolactinaemia. However, it seems necessary to determine the concentrations of 20α -dihydroprogesterone since it is the principal secretory product of the rabbit ovary. It may also, like progesterone, participate in the ovulatory process during this abnormal endocrine environment. Further study is in progress to clarify this point.

DNP-peptidase and BANA-hydrolase degrade native collagen at neutral and acid pH, respectively, and there is biochemical (Morales *et al.*, 1978; Fukumoto *et al.*, 1981) and morphological (Espey & Rondell, 1967; Okamura *et al.*, 1980) evidence to suggest that such enzymes are involved in the dissociation of the collagen matrix in the follicle wall at the time of ovulation. These enzymes increase about 7 h after hCG administration and then there is a decrease in the measurable amount of activity at 1–2 h before follicular rupture in the rabbit (Kawamura *et al.*, 1984; Okamura *et al.*, 1985) and human (Fukumoto *et al.*, 1981). The present results indicate that sulpiride-induced hyperprolactinaemia interferes with the normal preovulatory change in these collagen-degrading enzymes (e.g. production was less at 8 h than at 10 h after hCG in the follicles of the treated rabbits). Fukumoto *et al.* (1981) indicated that ovarian collagenolytic activity is mainly in the granulosa cells that is known to be affected by excess prolactin (McNatty *et al.*, 1974; Robyn *et al.*, 1977). Rondell (1974) has reported that progesterone may activate enzymes which increase the distensibility of the collagen framework of strips of sow follicles *in vitro*. Therefore, it is possible that prolactin exerts its anti-ovulatory effect by interfering with progesterone and/or collagenase metabolism in mature ovarian follicles.

Besser & Thorner (1975) found that chronic hyperprolactinaemia reduces oestrogen synthesis and causes hypogonadism. Our failure to find any differences in the ovarian output of oestrogen between sulpiride-treated and control rabbits (Japanese White strain) may be due to the shorter duration of treatment. In any event, it is doubtful that oestrogen is important in the mechanism of ovulation because the inhibition of oestrogen synthesis does not prevent ovulation from taking place in the in-vitro perfused rat ovary (Koos *et al.*, 1984). Likewise, it is doubtful that testosterone is a critical factor in ovulation (Mori *et al.*, 1977b; Wu *et al.*, 1977).

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