Effects of a 3β-hydroxysteroid dehydrogenase inhibitor on monocyte–macrophage infiltration into rat corpus luteum and on apoptosis: relationship to the luteolytic action of prolactin

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The administration of prolactin to hypophysectomized rats results in regression of the corpora lutea, accompanied by immune–inflammatory events such as infiltration of monocytes and macrophages. Recent reports indicate an autocrine role for progesterone during the lifespan of the corpus luteum. In the present study, an inhibitor of 3β-hydroxysteroid dehydrogenase, Trilostane, was used to investigate the hypothesis that a decrease in luteal tissue steroids precipitates the cascade of immune–inflammatory events leading to luteal regression in prolactin-treated hypophysectomized rats. Immature rats were induced to ovulate by administering eCG–hCG, and hypophysectomized on the day after ovulation (at 32 days of age). Rats were injected s.c. 9–11 days after hypophysectomy with (a) Trilostane (80 mg kg−1 day−1), (b) ovine prolactin (500 μg day−1), (c) Trilostane plus prolactin, or (d) vehicle. Plasma and luteal tissue progesterone and 20α-dihydroprogesterone (‘progestin’) were quantified; luteal tissue monocytes–macrophages and apoptotic nuclei were counted, and luteal wet mass was determined. Rats treated with prolactin alone showed the expected markers of luteal regression: decreased plasma progestin, increased numbers of monocytes–macrophages and apoptotic nuclei in luteal tissue, and decreased luteal wet mass; however, progestin concentration in luteal tissue was unchanged. Treatment with Trilostane reduced plasma and luteal tissue progestin, but did not result in an infiltration of monocytes–macrophages or increased numbers of apoptotic nuclei in the corpora lutea, or any change in luteal wet mass. Trilostane in combination with prolactin reduced plasma and luteal tissue progestin and produced the expected markers of regression, with the exception of luteal tissue mass, which remained unchanged. In conclusion, inhibition of steroidogenesis does not initiate luteal regression or augment prolactin-induced luteal regression in hypophysectomized rats. Prolactin-induced infiltration of monocytes–macrophages is not accompanied by a decrease in luteal tissue progestin, at least in the early stages of luteal regression.

Introduction

In mated rats, surges of prolactin twice a day stimulate the growth of the corpora lutea and promote the secretion of progesterone (Smith et al., 1976; Rothchild, 1981) whereas, in non-mated rats, a prolactin surge on the afternoon of pro-oestrus may promote regression of corpora lutea (Billeter and Flückiger, 1971; Wüttke and Meites, 1971). The requirement of prolactin for the regression of rat corpora lutea can be seen in hypophysectomized rats, in which regression of the corpora lutea is initiated when prolactin is administered (Malven and Sawyer, 1966; Taya and Greenwald, 1982). Immune–inflammatory events are involved in the luteolytic response to prolactin: administration of prolactin to hypophysectomized rats is associated with increased immunohistochemical staining for monocyte chemoattractant protein 1 (MCP-1) in luteal tissue, which is followed or accompanied by the accumulation of monocytes and macrophages in the corpora lutea, a decrease in plasma progestins, and a decrease in the mass of luteal tissue (Bowen et al., 1996, 1998).

Sex steroids and prolactin can affect the activities of immune–inflammatory cells (for review, see Miller and Hunt, 1996). Activated macrophages exposed to progesterone have a decreased capacity to produce nitric oxide (Miller and Hunt, 1996); oestrogen can inhibit expression of mRNA encoding JE–MCP-1 in mouse peritoneal macrophages (Frazier-Jessen and Kovacs, 1995), and prolactin can enhance the phagocytic ability of macrophages (Di Carlo et al., 1992; Chen and Johnson, 1993). Furthermore, oestrogen and prolactin receptors have been detected in macrophages...
(Gulshan et al., 1990; Gala and Shevach, 1993). In the present study, the relationship was examined between steroidogenesis and certain markers of luteal regression (including the infiltration of monocytes–macrophages) in the corpus luteum. Specifically, this study investigated whether a reduction of luteal steroidogenesis achieved by injection of Trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase (Potts et al., 1978), is a precipitating stimulus for infiltration of monocytes–macrophages and other regressive events in the corpora lutea.

Materials and Methods

Animals

Immature 29-day-old Sprague–Dawley female rats were injected s.c. with 50 iu equine chorionic gonadotrophin (eCG), and then injected s.c. with 25 iu human CG 56 h later to stimulate ovulation and corpus luteum formation. Injections were performed at Charles River Laboratories (Portage, MI) and the rats were hypophysectomized at 32 days of age (shortly after ovulation) by personnel at that facility (rats were anaesthetized with 43 mg ketamine kg⁻¹ and 8.7 mg xylazine kg⁻¹ i.p. obtained from Henry Schein Company (Port Washington, NY)). Rats were shipped to the authors’ laboratory 3 days later. The rats were provided with rat chow and 5% glucose in water ad libitum, and were given sliced oranges daily. They were housed in a room with controlled lighting (lights on 6:00–18:00 h) and controlled temperature.

Experimental design

In the first experiment, the hypophysectomized rats were divided into four groups and injected for 2 days with (a) Trilostane, (b) ovine prolactin, (c) Trilostane plus prolactin, or (d) vehicle. Trilostane or oil was injected s.c. every 4 h (80 mg kg⁻¹ day⁻¹), and prolactin was injected s.c. every 12 h (500 µg day⁻¹) on 9–11 days after hypophysectomy. Rats were killed by decapitation between 11:00 and 14:00 h on day 11. Trunk blood was collected from all rats and plasma was obtained for radioimmunoassay of steroids. The ovaries from each rat were removed immediately and frozen for immunohistochemistry. A second experiment was performed to determine whether this treatment with Trilostane caused any detectable changes in luteal tissue progesterone concentration. The same schedule and treatments were established: Trilostane (n = 8), prolactin (n = 6), Trilostane and prolactin (n = 7), and vehicle (n = 7). On day 11, the ovaries were removed, and ten corpora lutea were excised and weighed. The corpora lutea were snap frozen in liquid nitrogen and later homogenized for radioimmunoassay of tissue progesterone and 20α-dihydroprogesterone. In both experiments, the sella turcica was visually inspected in all rats for the presence of pituitary fragments. Rats with visible pituitary fragments were excluded from the experiment.

Hormones and reagents

Trilostane, a 3β-hydroxysteroid dehydrogenase inhibitor, was generously donated by Sanofi Winthrop Inc. (Malvern, PA). The Trilostane was suspended in canola oil at a final concentration of 6.7 mg ml⁻¹. The suspended Trilostane was refrigerated at 4°C and used within 1 week. The suspension was stirred thoroughly before each injection. Ovine prolactin (NIIDK-oPRL-20, lot number AFP10677C) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases of the NIH (Bethesda, MD). The prolactin was diluted in 0.15 mol NaCl l⁻¹, 0.03 mol NaHCO₃ l⁻¹ and 0.1% BSA to a final concentration of 1.25 mg ml⁻¹ and pH of 8.2–8.6. Diluted prolactin was stored at 4°C and used within 1 week. The monoclonal antibody against rat monocytes–macrophages (clone ED1) was obtained from Chemicon International Inc. (Temecula, CA). The specificity of this antibody for rat monocytes–macrophages has been reported by Dijkstra et al. (1985). Biotinylated secondary antibody to mouse immunoglobulin was obtained from Vector Laboratories (Burlingame, CA).

Immunohistochemistry

One ovary from each rat was frozen in ornithine carbamyl transerase compound (Miles Laboratories, Inc., Elkhart, IN). Frozen tissue sections (6–8 µm) were air-dried, fixed in 95% ethanol for 10 min and then placed in 0.3% (v/v) H₂O₂ in methanol at 4°C for 15 min to quench endogenous peroxidase activity. The tissue sections were then rinsed three times (5 min each) in PBS–1% BSA and then incubated with 10% normal goat serum for 30 min at room temperature. The sections were rinsed again in PBS–1% BSA and then incubated with a monoclonal antibody against rat monocytes–macrophages (1:200 dilution for 45 min at room temperature). The sections were rinsed in PBS–0.1% BSA and then exposed to biotinylated goat anti-mouse immunoglobulin (1:100 dilution for 30 min at room temperature). The antigen-antibody complex was detected using an avidin–biotin–peroxidase (ABC) kit (Vector) and 3-amino-9 ethylcarbazole (AEC) as the substrate. The sections were counterstained with haematoxylin, rinsed in distilled water, and dipped in Scott’s tap water before mounting with aqueous mounting medium. Non-specific staining was assessed by replacement of primary or secondary antibodies with the appropriate serum, and was undetectable in all instances.

Detection of apoptosis in situ

Nuclei showing DNA fragmentation were detected by in situ hybridization using the ApoTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Frozen sections were air-dried and fixed in 10% (v/v) neutral buffered formalin for 10 min. The sections were then rinsed in two changes of PBS for 5 min each before being post-fixed in ethanol:acetic acid (2:1) for 5 min at 20°C. After post-fixing, sections were rinsed again in two changes of PBS, and then quenched in 3% (v/v) H₂O₂ in PBS for 5 min at room temperature.
temperature. Sections were rinsed in PBS, and equilibration buffer from the kit was applied to cover each section. The buffer was blotted and replaced with a solution of terminal deoxynucleotide transferase (TdT). The sections were incubated with this enzyme for 1 h at 37°C to allow for tagging of 3' DNA ends with digoxigenin residues. The sections were next placed in stop buffer for 10 min, and then rinsed with PBS and incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min at room temperature. After rinsing in PBS, sections were incubated for 6 min with diaminobenzidine (DAB) substrate, washed in three changes of distilled water for 1 min each, and then in distilled water for 5 min. The sections were counterstained with methyl green for 10 min, washed three times in distilled water, three times in 100% butanol, and three times in xylene before mounting. Labelling of atretic follicles within the whole ovarian sections served as an internal positive control. Negative controls, in which the TdT enzyme solution was replaced with water, were run in every batch of slides stained, and this replacement completely eliminated staining.

Radioimmunoassays

Rats were decapitated and trunk blood was collected into heparinized tubes and then centrifuged at 1740 g for 20 min to obtain plasma. The plasma was then frozen in liquid nitrogen and stored at –20°C. Isolated corpora lutea were homogenized in 1 ml PBS and the homogenate was frozen in liquid nitrogen and stored at –20°C. Plasma and tissue homogenates were assayed for progesterone and 20α-dihydroprogesterone according to the methods described by Elbaum et al. (1975) and Bender et al. (1978), respectively. Results are expressed as ‘progestin’, that is, the combined values for progesterone and 20α-dihydroprogesterone per ml of plasma or per mg of luteal tissue.

Statistical analysis

One-way analysis of variance was performed for each variable to determine whether there were significant differences among the groups. After this analysis, differences between individual groups were identified using the Bonferroni multiple comparisons test. Plasma and luteal progestin values were log-transformed to normalize variance before statistical analysis.

Quantitation of macrophages and apoptotic nuclei

The number of luteal macrophages per high power field was determined by visual observation of immunodetectable cells using coded slides and a light microscope with a × 45 objective. For each rat, positively stained macrophages were counted in 1–3 high-power fields in each of the multiple corpora lutea examined, and these counts were averaged to obtain a single number of macrophages per high-power field (n = 1). Coded slides were also used for the quantitation of apoptotic nuclei. For each rat, apoptotic nuclei were counted in multiple high-power fields in each of the multiple corpora lutea examined, and these counts were averaged to obtain a single number of apoptotic nuclei per high power field (n = 1).

Results

Plasma progestin concentration (Expt 1)

Plasma concentration of progestin (mean ± SEM) was significantly lower in rats that received Trilostane and prolactin (17.6 ± 3.6 nmol l⁻¹), or Trilostane (25.6 ± 5.6), than it was in rats treated with either prolactin (120.8 ± 18.5) or vehicle (320.5 ± 32.7) (P < 0.05). Furthermore, plasma concentration of progestin in prolactin-treated rats was lower than it was in controls (P < 0.05; Fig. 1). The ratio of 20α-dihydroprogesterone:progesterone was approximately 6 for all rats receiving Trilostane (with or without prolactin), and approximately 25 for all other rats.

Luteal tissue progestin concentration (Expt 2)

Luteal tissue progestin concentration was significantly lower in rats that received Trilostane and prolactin (9.8 ± 2.1 nmol per mg luteal tissue), or Trilostane (4.9 ± 0.7), compared with that in rats treated with either prolactin (59.9 ± 10.6) or vehicle (42.1 ± 4.1) (P < 0.05).

Ovarian and luteal wet mass (Expts 1 and 2)

No differences were observed in whole ovarian wet masses (Expt 1; data not shown). In Expt 2, the wet masses of the corpora lutea were: 0.51 ± 0.05 (Trilostane and prolactin);
0.54 ± 0.06 (Trilostane); 0.40 ± 0.03 (prolactin); 0.62 ± 0.05 mg (vehicle). The mean wet masses of corpora lutea for prolactin- and vehicle-treated rats were significantly different (P < 0.05).

### Luteal monocytes–macrophages (Expt 1)

The number of monocytes–macrophages per high power field in the corpora lutea of the different groups are shown (Fig. 2). Monocytes–macrophages accumulated in the corpora lutea of prolactin-treated and Trilostane plus prolactin-treated rats after 48 h of treatment. The numbers of luteal monocytes–macrophages (mean ± SEM) were significantly higher (P < 0.05) in Trilostane and prolactin-treated animals (44.6 ± 2 per high power field) and in prolactin treated rats (47.5 ± 1.9) compared with those in Trilostane-treated (16.1 ± 1.6) and vehicle-treated rats (16.0 ± 1.4).

### Luteal apoptotic nuclei (Expt 1)

The appearance of apoptotic nuclei in ovarian sections from each group are illustrated (Fig. 3). Apoptotic nuclei (brown) are more prevalent in the corpora lutea of rats treated with Trilostane and prolactin (Fig. 3a or prolactin (Fig. 3c) than in the corpora lutea of rats treated with Trilostane (Fig. 3b) or vehicle (Fig. 3d). Atretic follicles in the ovarian sections served as an internal positive control. The cross-sections of such follicles, containing large numbers of apoptotic nuclei, are illustrated (Fig. 3b-d). The number of apoptotic cells per high power field in corpora lutea was quantified (Fig. 4). Overall, treatment with prolactin resulted in a significant (P < 0.05) increase in the number of apoptotic cells. However, fewer apoptotic cells were observed in the corpora lutea of Trilostane and prolactin-treated rats than in the corpora lutea of prolactin-treated rats (2.2 ± 0.4 and 5.0 ± 1.0 per high power field, respectively; P < 0.05). Treatment with Trilostane alone did not cause any detectable increase in the incidence of apoptosis (Trilostane versus vehicle: 0.3 ± 0.1 versus 0.4 ± 0.1 per high power field).

### Discussion

The present study took advantage of the functional and morphological stability of the corpora lutea in hypophysectomized rats, in which the corpora lutea continue to produce 20α-dihydropregesterone and low amounts of progesterone for weeks (Taya and Greenwald, 1982). These corpora lutea remain in this stable condition presumably because of the absence of prolactin, the hormone thought to be primarily responsible for normal regression of corpora lutea in rats (Billeter and Flückiger, 1971; Wüttke and Meites, 1971). When prolactin is administered to hypophysectomized rats, the corpora lutea undergo a swift chain of events leading to regression of the corpora lutea (Malven and Sawyer, 1966; Taya and Greenwald, 1982). This regression is accompanied by an infiltration of monocytes–macrophages, and an increase in immunodetectable MCP-1 in corpora lutea (Bowen et al., 1996). The final signals and pathways leading to regression in this case have not been fully delineated, but one means of investigating them is through the steroids produced within the corpora lutea. Luteal steroids may play a role either in the recruitment of monocytes–macrophages or in the responses of recruited monocytes–macrophages, which change activity in response to steroids (Guilshan et al., 1990; Loy et al., 1992; Chao et al., 1995; Frazier-Jessen and Kovacs, 1995; Miller and Hunt, 1996). Progesterone may have an autocrine role in the rat corpus luteum. Sugino et al. (1997) used a rat luteal cell line, and normal rat luteal tissue in vitro to determine that either progesterone or dexamethasone inhibited the expression of mRNA encoding 20α-hydroxysteroid dehydrogenase. Hypophysectomized rats were treated with RU486 in an attempt to block the hypothesized actions of progesterone in the corpus luteum; however, no significant effect on plasma progestins was observed (Arakawa et al., 1990). Moreover, the use of RU486 in vivo has not revealed a consistent autocrine role for progesterone independent of changes in pituitary prolactin secretion (Ultenbroek et al., 1995). Nevertheless, the hypothesis that progesterone has an autocrine role in the corpus luteum (Rothchild, 1981) led to the proposal that the series of regressive changes stimulated by prolactin is mediated through reduced ovarian steroids.

Administration of Trilostane effectively reduced the plasma concentration of progestin, and the 3β-hydroxysteroid dehydrogenase inhibitor effectively reduced the luteal tissue concentration of progestin. However, despite the reduction of steroids, no evidence of regressive changes was detected in the corpora lutea of the rats treated with only Trilostane (the luteal wet mass, number of luteal monocytes–macrophages, and number of apoptotic cells in corpora lutea were not different from those in the vehicle-treated rats). These results are set against the background of the overt
regressive effects of prolactin treatment: reduced luteal wet mass, increased number of monocytes–macrophages and increased incidence of apoptosis in luteal tissue. These observations of the regressive effects of prolactin are consistent with previous results (Bowen et al., 1996, 1998), and with the reported regressive effects of prolactin during the rat oestrous cycle (Matsuyama et al., 1996; Gaytán et al., 1997; Bowen et al., 1999). Thus, the reduction of luteal steroids through inhibition by Trilostane is not an effective stimulus on its own for monocyte–macrophage recruitment or for induction of regression through the apoptosis pathway.

One interpretation of the results of the present study is that the corpora lutea of rats treated with Trilostane are resistant to the stimuli for regression, that is, they are resistant to the action of prolactin. Indeed, some blunting of the effects of prolactin by Trilostane was observed. Rats treated with Trilostane and prolactin did not show reduced luteal wet masses, and luteal tissue from these rats had a lower incidence of apoptosis compared with the corpora lutea of rats treated with prolactin only. However, the numbers of monocytes–macrophages did not differ between these two groups, and the incidence of apoptosis in Trilostane–prolactin treated rats was higher than it was in either the vehicle- or Trilostane-treated animals. This finding indicates that regressive changes had commenced when the rats were killed.

An assumption underlying this entire study was that the regressive actions of prolactin would be accompanied by a reduction in luteal tissue steroids, also reflected by
Trilostane or oil was injected s.c. every 4 h (80 mg kg⁻¹ day⁻¹), and prolactin was injected s.c. every 12 h (500 μg; hypophysectomized rats treated with Trilostane and prolactin). Corpora lutea. This finding is consistent with other studies detecting a diminution of the measured progestins in isolated corpora lutea of prolactin-treated, hypophysectomized rats in the intact cyclic rat using 2-Br-α-ergokryptine (CB154) (Experiencia 27 464–465).

Fig. 4. Numbers of apoptotic nuclei in corpora lutea of hypophysectomized rats treated with Trilostane and prolactin (n = 6). Trilostane (n = 8), prolactin (n = 6) or vehicle (n = 6). Trilostane or oil was injected s.c. every 4 h (80 mg kg⁻¹ day⁻¹), and prolactin was injected s.c. every 12 h (500 μg; hypophysectomized rats treated with Trilostane and prolactin) on days 9–11 after hypophysectomy. Values are the mean ± SEM. Values with different letters are significantly different (P < 0.05).

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Inhibition of steroidogenesis and luteal regression in rats


