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Follicle Stimulating Hormone, Luteinizing Hormone and Testicular Leydig Cell Responses to Estradiol Immunization in Ile-de-France Rams

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Active immunization of Ile-de-France rams against estradiol (E_2) resulted in the production of E_2-neutralizing antibodies and an elevation in the plasma concentrations of FSH, LH, and testosterone. The presence of E_2 antibodies did not affect the testosterone metabolic clearance rate, indicating that the immunization-mediated 10-fold increase in plasma testosterone was the result of a 10-fold increase in testicular testosterone production. Testis weights, as well as nuclear and cytoplasmic volumes of individual peritubular and perivascular Leydig cells, were greater in E_2-immunized rams than in albumin-immunized controls. Leydig cell numbers were not affected by treatment. The E_2 antibodies were capable not only of neutralizing the inhibitory effects of endogenous E_2 on gonadotropin levels in intact rams, but were able to block the effects of exogenously administered E_2 on their FSH and LH secretory response to castration. It is concluded that circulating E_2 in the ram is involved in pituitary-testicular endocrine homeostasis and that E_2 immunoneutralization can be employed to enhance testosterone secretion in this species.

Key words: estradiol immunoneutralization, gonadotropins, testosterone secretion, ram testes.

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Estradiol-17β (E_2) in peripheral blood results from gonadal (Payne et al, 1976; Dorrington et al, 1978) and extragonadal (Longcope et al, 1978; Worgul et al, 1981) production. Whereas intratesticular estrogen acts locally to regulate steroid biosynthesis in Leydig cells (Hsueh et al, 1978; Melner and Abney, 1980; Moger, 1980), peripheral estrogens can affect Leydig cell function indirectly via their feedback actions on the hypothalamic-pituitary axis (Swerdloff and Odell, 1968; deJong et al, 1975).

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In male sheep, E_2 is a potent regulator of gonadotropin secretion (Riggs and Malven, 1974; D'Occhio et al, 1983), presumably by acting through specific receptors at the levels of the hypothalamus (Pelletier and Caraty, 1981) and/or the pituitary (Thieulant and Pelletier, 1979; Glass et al, 1984). The physiologic importance of picomolar concentrations of circulating E_2 in the feedback regulation of LH secretion in intact rams has been demonstrated only recently (Schanbacher, 1984). Active immunization of Suffolk rams against E_2 elicited a two- to three-fold increase in mean serum LH concentrations and a parallel increase in mean serum testosterone concentrations. These data suggest that circulating E_2 plays a physiologic role in the regulation of pituitary LH secretion and that E_2 immunoneutralization can be employed to stimulate the reproductive-endocrine axis of rams. The following study was undertaken in the Ile-de-France breed to extend our previous findings regarding estradiol immunization effects in the domestic ram. The parameters assessed included: 1) FSH secretion; 2) testosterone production and metabolic clearance rates; 3) Leydig cell morphology; and 4) antibody effects on exogenous as well as endogenous E_2.

Materials and Methods

Animals and Immunization

Four-year-old Ile-de-France rams weighing approximately 80 kg were assigned to the following study. All

animals were kept at the INRA research facility near Nouzilly and were fed alfalfa hay, wheat straw, and grain supplement as required for body weight maintenance. Water, salt, and mineral supplement were provided *ad libitum*. Experimentation began in October at the time of primary immunization and extended into the nonbreeding season, a time when testosterone levels generally are suppressed.

Rams were randomly assigned to one of two groups. Five rams were actively immunized against the carrier protein bovine serum albumin (BSA; 1 mg) while each of a second group of five rams was immunized with 1 mg of an E_2-6-CMO-BSA conjugate (E_2:BSA; Steraloids, Inc., Wilton, NH). Antigens were dissolved in 1 ml saline and then emulsified with 1 ml Freund's complete adjuvant as described previously (Schanbacher, 1984). Injections were made in 20 or more subcutaneous sites over the ribs. BSA and E_2:BSA rams were given the respective antigens as booster injections (1 mg each) in December and February.

Experiment 1

BSA- and E_2:BSA-immunized rams were sampled in April (ovine nonbreeding season) via jugular venipuncture at 20-minute intervals for 8 hours. Plasma from these samples were stored frozen at −20 C for subsequent determination of E_2 antibody binding titer and concentrations of FSH, LH, and testosterone. All ten rams were subsequently castrated under pentothal anesthesia. Pieces of testes were fixed in Bouin Holland's solution (right testis) or in glutaraldehyde:collidine buffer followed by osmium (left testis). Ten-micron sections were stained by the Feulgen reaction and counterstained with Alcian blue for histologic assessment of peritubular and perivascular Leydig cells (Lunstra and Schanbacher 1987). The area of individual cells, total number of cells, and total volume of these cells were determined by morphometric means (Hochereau-de Reviers et al, 1979). Fifteen days postcastration, when circulating testosterone levels were not detectable, estimates of testosterone metabolic clearance rates for BSA- and E_2:BSA-immunized animals were ascertained. Each animal was injected intravenously with 1 mg of testosterone dissolved in 2 ml of 10% ethanolic saline at 1000 hours. Plasma samples were collected at 5-minute intervals starting 10 minutes after injection through 60 minutes and again at 90 and 120 minutes. The natural log transformation of plasma testosterone concentrations was used to calculate the decay constant ($-\alpha$) used in derivation of the testosterone metabolic clearance rate (Terqui, 1978). The equation, $r = Ae^{-\alpha t}$, simplifies to $MCR_T = \alpha/A$, where r is testosterone in fraction of the injected dose per liter of plasma, t is time expressed in minutes, and A the fractional dose intercept. Testosterone daily production rates of immunized rams were computed from the product of mean plasma testosterone concentrations and the testosterone metabolic clearance rate (Baird et al, 1969).

Experiment 2

The BSA- and E_2:BSA-immunized rams were implanted with a 6-cm Silastic capsule containing estradiol (Schanbacher, 1984) at the time of castration to assess their susceptibility to E_2 negative feedback. This dose of E_2 is known to suppress LH secretion, especially during the nonbreeding season. The postcastration gonadotropin response, including FSH and LH, was determined in plasma samples collected on days 4, 7, and 11. Blood samples were taken again via jugular venipuncture at 20-minute intervals, but for only 3 hours (0900–1200 hours) on each of the respective days. Plasma LH concentrations were determined in all samples, whereas FSH and E_2 antibody binding titers were determined in plasma pools prepared from samples of each animal. E_2 implants were removed from all animals after blood sampling on day 11.

Hormone Assays and E_2 Antibody Titers

Plasma FSH (Blanc and Poirier, 1979) and LH (Pelletier et al, 1982) were determined by double antibody radioimmunoassay procedures described previously. Determinations were made in duplicate using 100 μl of plasma. Assay sensitivities and intraassay coefficients of variation were: FSH, 1 ng/ml HG-FSH 225 (35 × NIH-FSH-S3) and 7.5%; LH, 300 pg/ml LH-M-3 (1.8 × NIH-LH-S1) and 8.9%.

Plasma testosterone concentrations were also determined by double antibody radioimmunoassay (Garnier et al, 1978) but in duplicate 50-μl ethylacetate:cyclohexane (1:1, v:v) extracts. Extraction efficiency using 10 volumes of extracting solvent averaged 92 ± 6%. The minimal detectable amount of testosterone was 200 pg/ml and the intraassay coefficient of variation was 11.8% for a plasma pool containing 2.5 ng/ml.

E_2 antibody titers were determined by a charcoal precipitation assay (Schanbacher, 1984) and the results expressed as the percentage of radiolabeled E_2 specifically bound in 1000-fold plasma dilutions. In brief, diluted plasma was incubated overnight at 4 C with 20,000 dpm (18 pg) tritium-labeled E_2 (Amersham Radiochemical Centre, England) and free and bound tracer were separated the next day by dextran-treated charcoal. The supernatant (bound fraction) was counted by liquid scintillation spectroscopy.

Morphometric Analyses

Intertubular tissue relative volume was assessed on 20 fields per left testis with an ocular reticule integrator (25 points) at a × 256 magnification. Leydig cell relative volume was assessed similarly at a × 1,600 magnification. Total intertubular and Leydig cell volumes were then calculated. Mean cross sectional areas of Leydig cells or Leydig cell nuclei were measured with an automatic planimeter on at least 20 peritubular cells per testis adjacent to stage 8 tubules of the seminiferous epithelium cycle (Roosen-Runge and Giesel, 1950). Perivascular Leydig cells were measured independently of the stages of the seminiferous epithelium.

Assuming there were similar numbers of peritubular and perivascular Leydig cells, mean Leydig cell volume and total number of Leydig cells per testis were calculated. Perivascular Leydig cell cross sectional areas were also measured on four glutaraldehyde-fixed testes per treatment group. Two-μm semithin sections, stained with Toluidine Blue, were prepared and Leydig cells were measured only when their nuclei contained an equatorial nucleolus.

TABLE 1. (Experiment 1). Mean plasma FSH, LH and Testosterone Concentrations and Testosterone Metabolic Clearance Rate and Production Rate Estimates after Immunization Against Bovine Serum Albumin or an Estradiol:BSA Conjugate in Mature Ile-de-France Rams*

	BSA Rams	E_2:BSA Rams
E_2 binding titer (B/Bo at 1:1000)	< 0.5	39.8 ± 5.2
FSH		
Mean conc. (ng/ml)	1.9 ± 0.2	4.3 ± 0.9†
LH		
Mean conc. (ng/ml)	1.4 ± 0.5	3.3 ± 0.6†
Peak interval (min)	336 ± 59	110 ± 14†
Peak amplitude (ng/ml)	3.4 ± 1.2	8.2 ± 1.3†
Testosterone		
Mean conc. (ng/ml)	2.5 ± 0.3	23.7 ± 4.9†
MCR (l/d)‡	1959 ± 51	1886 ± 47
PR (mg/d)‡	6.3 ± 0.7	64.2 ± 3.7†

*Mean plasma hormone concentrations (± SEM, n = 5) were calculated from an 8-hour intensive bleed in April (primary immunizations were given in October with booster injections in December and February).

†Significantly different from BSA control value, $P < 0.01$.

‡MCR = metabolic clearance rate.

PCR = daily production rate.

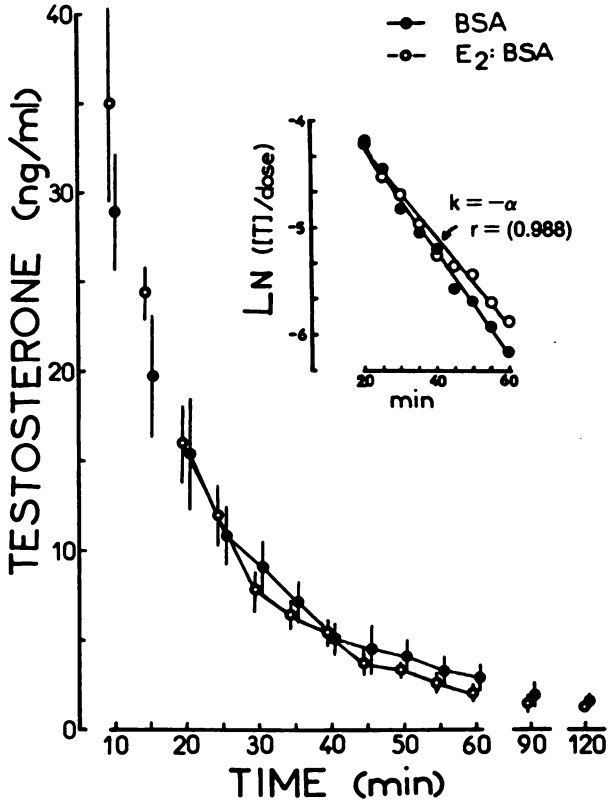

Fig. 1. Disappearance of immunoreactive testosterone in plasma of acutely castrated, albumin- (BSA) and estradiol: albumin-(E_2:BSA) immunized rams after a 1-mg intravenous bolus injection at time zero. Metabolic clearance rates were determined from the linear decay curves observed between 20 and 60 minutes (inset) to be similar for BSA- and E_2:BSA-immunized rams.

Statistical Analyses

All treatment means for BSA- and E_2:BSA-immunized rams were compared utilizing student's *t*-test (Freund et al, 1960).

Results

Experiment 1

Immunization of mature Ile-de-France rams against E_2-6-CMO-BSA resulted in the production of antibodies capable of binding tritiated E_2 (Table 1). Whereas approximately 40% of the tracer was bound in E_2:BSA ram plasma diluted 1000-fold, plasma from BSA-immunized rams bound less than 0.5% of the E_2 tracer. Competitive inhibition curves yielded relative cross reactivities of 0.8% for testosterone and less than 0.1% for dihydrotestosterone.

E_2:BSA-immunized rams were found to have elevated plasma FSH, LH, and testosterone when compared with BSA-immunized controls (Table 1). Plasma LH and testosterone were secreted episodically in all rams with the frequency and amplitude of the LH secretory episodes being greater in E_2:BSA-immunized rams. The linear decay curves of exogenously administered testosterone between 20 and 60 minutes were nearly identical (Fig. 1), suggesting that disappearance of testosterone from the plasma compartment of BSA- and E_2:BSA-immunized rams was the same. Indeed, the calculated metabolic clearance rate for testosterone of E_2:BSA rams was not significantly different from that of BSA-immunized controls. Consequently, the observed 10-fold increase in plasma testosterone of E_2:BSA-immunized rams reflected a 10-fold increase in testosterone daily production rate (Table 1).

Testis weights were significantly heavier in E_2:BSA-immunized rams. Light microscopic inspection revealed an increase in nuclear and cytoplasmic volume occupied by individual Leydig cells of these rams (Table 2). This increase in cell size was observed for both peritubular and perivascular Leydig cells. An increase in perivascular Leydig cells was also observed in semithin sections from E_2-immunized rams (64.5 ± 4.5 vs. 85.4 ± 12.5 μm^2). The total volume of Leydig cells per testis was significantly increased in

TABLE 2. (Experiment 1). Testis Weight and Leydig Cell Size and Number in Mature Ile-de-France Rams after Immunization Against Bovine Serum Albumin or an Estradiol:BSA Conjugate*

	BSA Rams	E_2:BSA Rams
Testis weight (gm)	173.4 ± 11.4	219.3 ± 12.4†
Cross sectional area (μm^2)		
Peritubular Leydig cells	37.9 ± 2.2	49.0 ± 1.7†
Perivascular Leydig cells	45.9 ± 2.1	57.9 ± 3.6†
Perivascular Leydig cell nuclei	21.6 ± 0.5	25.7 ± 0.9†
Leydig cell number/testis (10^8)	13.1 ± 0.34	12.3 ± 0.6
Leydig cell total volume/testis (cm^3)	2.62 ± 0.13	3.66 ± 0.31†

*Mean ± SEM, n = 5) determined from morphometric analyses of testis tissue collected in April.

†Significantly different from BSA control value, $P < 0.01$.

E_2-immunized animals although the calculated number of Leydig cells per testis was not significantly different between groups.

Experiment 2

While a small but significant ($P < 0.05$) FSH rise was observed in BSA-immunized, E_2-implanted animals following castration, a pronounced postcastration rise in both plasma FSH and LH was observed in E_2:BSA-immunized, E_2-implanted animals possessing circulating E_2 antibodies (Table 3). The acute gonadotropin response to castration in the former group and the high plasma levels of FSH and LH achieved by day 11 suggest that the biologic activity of exogenous E_2 was neutralized by the circulating antibody. Excess binding (E_2 antibody) was observed in the plasma of E_2:BSA rams during the three sampling periods despite their continuous exposure to E_2.

Discussion

Active immunization of mature Ile-de-France rams against E_2-6-CMO-BSA resulted in the production of circulating antibodies to E_2. This method of neutralizing the biologic activity of a steroid (Nieschlag et al, 1975), and of E_2 in particular (Nishihara and Takahashi, 1983; Schanbacher, 1984), has proven itself useful in determining the biologic actions of endogenous circulating E_2. Similarly, the action of exogenous E_2 can also be blocked as shown in Experiment 2 of the present study. When compared with passive immunization, active immunization has the advantage of requiring small amounts of foreign protein (i.e., antigen vs. gamma globulin) and prolonged *in vivo* antibody production can produce a condition of chronic hormone deficiency.

Immunoneutralization of circulating E_2 in Ile-de-France rams resulted in elevated plasma FSH, LH, and testosterone concentrations. The increase in LH and testosterone secretion in E_2-immunized Ile-de-France rams confirms similar observations in E_2-immunized male rats (Nishihara and Takahashi, 1983), E_2-immunized Suffolk rams (Schanbacher, 1984) and adult crossbred rams passively immunized with an antisera raised against E_2 (Sanford, 1985). Curiously, passive immunization of Merino ram lambs against E_2 stimulated growth of the testis but did not affect mean plasma levels of FSH, LH, and testosterone (Land et al, 1981). Increased plasma testosterone appears to be the result of gonadotropin-stimulated synthesis and secretion as reflected in the 10-fold increase of the testosterone daily production

TABLE 3. (Experiment 2). Mean Plasma FSH and LH Concentrations after Castration and Estradiol Implant Administration in Bovine Serum Albumin- and Estradiol:BSA-immunized Ile-de-France Rams*

	Days Postcastration:Implantation			
	-	4	7	11
E_2 binding titer (B/Bo at 1:1000)				
BSA	< 0.5	< 0.5	< 0.5	< 0.5
E_2:BSA	36.0 ± 5.8†	33.5 ± 5.9†	34.0 ± 6.3†	32.9 ± 7.8†
FSH (ng/ml)				
BSA	1.7 ± 0.2	4.0 ± 0.2	4.2 ± 0.1	6.3 ± 1.7
E_2:BSA	4.5 ± 0.4†	17.4 ± 1.7†	24.4 ± 0.5†	26.8 ± 3.2†
LH (ng/ml)				
BSA	1.4 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.2
E_2:BSA	3.1 ± 0.7†	5.8 ± 0.3†	7.1 ± 0.5†	7.9 ± 0.5†

*Mean ± SEM, n = 5.

†Significantly different from the corresponding BSA control value, $P < 0.01$.

rate. Although the E_2 antibody cross-reacted slightly with testosterone and dihydrotestosterone, the testosterone metabolic clearance rate was unaffected by immunization. Nishihara and Takahashi (1983) drew the same conclusion in E_2-immunized male rats. In their study, active immunization employed the same antigen as that used in the present study, but the testosterone metabolic clearance rate and the testosterone daily production rate were determined utilizing the method of constant infusion of tritiated testosterone. From these results, it must be concluded that interactions between circulating E_2 antibody and testosterone are negligible and that testosterone production is increased.

Observations that concurrent increases in testosterone and plasma LH levels occur in rams immunized against E_2 lead us to conclude that E_2 action on the brain, rather than testosterone and its aromatization by the brain, accounts for a major part of steroid feedback control of LH secretion. Therefore, it appears that testicular or peripheral E_2 production is of greater physiologic significance to the ram than E_2 production derived from central aromatization. Results of this E_2 immunization experiment also suggest chronic regulation of FSH secretion by E_2. It has been reported that E_2 circulates in intact rams (Schanbacher and Ford, 1976; Sanford et al, 1982) and that E_2 implants can produce dose-dependent suppression of serum FSH in castrate rams (Schanbacher, 1979). However, the biologic significance of E_2-regulated FSH secretion remains uncertain. Perhaps low levels of E_2 synergize with testicular inhibin to regulate FSH. Interestingly, Barenton and Pelletier (1983) and Barenton et al (1983) showed that E_2 production by the ram testis parallels the onset of spermatogenesis and testosterone secretion.

The susceptibility of gonadotropin secretion (both LH and FSH) to E_2 feedback and the efficacy by which circulating E_2 antibody neutralizes the biologic actions of E_2 were demonstrated in Experiment 2. Utilizing the acutely-castrated, E_2-treated ram model (Schanbacher, 1984), we observed a maintenance of plasma LH levels in BSA-immunized rams comparable to that of precastration or normal intact ram values for 11 days (until the E_2 implants were removed). Plasma FSH levels during the same postcastration:implant sampling period were only moderately increased over intact levels, suggesting that E_2 may also participate in the feedback regulation of FSH secretion. In contrast, both plasma LH and FSH were increased markedly in E_2:BSA rams (Table 3). We conclude from these data that the circulating E_2 antibodies are capable of neutralizing (inactivating) exogenous as well as endogenous sources of estrogen.

Testosterone secretion in E_2:BSA-immunized rams was markedly enhanced and suggests that E_2 immunization may provide a means for elevating circulating androgen levels, particularly during the nonbreeding season (Schanbacher, 1984). Elevated peripheral testosterone levels were associated with hypertrophy of both peritubular and perivascular Leydig cells. This response to E_2 immunization may have been mediated peripherally (indirectly) as a result of increased gonadotropin release or perhaps, via circulating E_2 antibody, directly upon the testis. For example, the high affinity E_2 antibodies within the testicular vascular system may quickly and efficiently neutralize E_2 of testicular origin to remove its possible inhibitory influence on steroidogenic enzymes within the testis. The presence of estrogen receptors in Leydig cells (Brinkman et al, 1972; van Beurden-Lamers et al, 1974) and the observed inhibitory effects of E_2 on testicular steroid biosynthesis *in vivo* (Hsueh et al, 1978; Kalla et al, 1980; Nozu et al, 1981) and *in vitro* (Brinkman et al, 1980) support an existing hypothesis for possible direct effects of E_2 on the testis.

In conclusion, plasma FSH, LH, and testosterone levels are increased in mature Ile-de-France rams during the spring, or nonbreeding season, following E_2 immunization. These responses could prove beneficial for several facets of male reproductive function, including spermatogenesis, sperm cell viability, and libido.

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