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Fetal gonadotropins
hypothalamus

Müllerian inhibiting substance
newborn testes

Inhibition of Müllerian Inhibiting Substance Secretion by FSH

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Summary

To evaluate the role of gonadotropins in the control of Müllerian Inhibiting Substance (MIS) secretion, pregnant rats were injected with rabbit antiserum against luteinizing hormone releasing hormone (LHRH), and their pups replaced with follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG). The LHRH antiserum (LHRH-AS) was given at 13 and 20 days of gestation. Control dams were injected with an equal volume of normal rabbit serum. The male pups from mothers treated with LHRH antiserum were given 5 daily s.c. injections of the FSH, hCG, or vehicle. The male pups from mothers treated with normal rabbit serum were given vehicle s.c. Testicular fragments of 6 day old pups born to mothers treated with LHRH antiserum during pregnancy showed an increase relative to controls in MIS activity in a graded organ culture bioassay system (grade 3.4 ± 0.3 *vs.* 2.3 ± 0.2) ($P < 0.01$). FSH given to pups from mothers treated with LHRH antiserum reduced testicular MIS secretion compared to vehicle treated pups from the same mothers (grade 2.3 ± 0.2 *vs.* 3.4 ± 0.3) ($P < 0.01$). Thus, postnatal injections of FSH after immunologic blockade of gonadotropins *in utero* reduced MIS activity of the testes to the same level found in testes of 6 day control pups (grade 2.3 ± 0.2 *vs.* 2.3 ± 0.2). In contrast, MIS activity remained high despite postnatal hCG injection in pups born to mothers given LHRH-AS (grade 3.4 ± 0.4 *vs.* 3.4 ± 0.3). These studies suggest that secretion of MIS is dependent on normal hypothalamic secretion of LHRH and may be inhibited by FSH.

Speculation

FSH inhibits MIS secretion.

MIS and testosterone are the principal hormones which govern the embryonic development of male genitalia. MIS, first described in mammals by Jost (17, 18) causes regression during midgestation of the Müllerian ducts of the male rat (26, 27). MIS is secreted by the testis from day 14 of fetal life to birth and, in progressively lesser concentrations, until day 21 of postnatal life. Testosterone induces development of the Wolffian duct into the vas deferens, seminal vesicle and epididymis (8); and its active metabolite, 5-dihydrotestosterone, stimulates the urogenital sinus, genital tubercle, genital folds, and genital swellings to form prostate, glans, penis, and scrotum (8).

It is uncertain whether MIS is secreted autonomously or under extragonadal influences. Studies of the pituitary control of MIS have been inconclusive and conflicting. Maraud *et al.,* (23, 24) demonstrated an increase in MIS activity of the 2-month-old chick testes after hypophysectomy at 1 month. However, Groenendij-Huijbers and Burggraaff (12) noted a spontaneous return of MIS activity in the testes of the 4-month old chick. However, rats hypophysectomized at 20 days of age failed subsequently to demonstrate an elevation in testicular Müllerian inhibiting substance (11). Neither prolactin (Donahoe *et al.*, unpublished data), nor placental fragments added to the *in vitro* culture influenced MIS activity.

Previously, we demonstrated that an antiserum to luteinizing hormone releasing hormone (LHRH-AS) blocked endogenous LHRH secretion in the neonatal rat (3–5). Both serum concentration and pituitary content of FSH and luteinizing hormone (LH) were reduced after injection of the LHRH antiserum (2, 19). Furthermore, an increase in MIS occurred after injection of LHRH antiserum, which suggested that FSH and/or LH inhibited MIS secretion from the testes (6).

In this study, pregnant rats were treated with LHRH antiserum and their male pups treated postnatally with FSH or hCG in order to determine which of the pituitary gonadotropins influenced MIS secretion.

MATERIALS AND METHODS

ANIMALS

Adult rats were obtained from Holtzman Laboratories. Females in estrus had been caged with males overnight and those with sperm positive vaginal smears on the next morning were used for study. That morning was recorded as day ½ of pregnancy. The animals were housed in a temperature-controlled room (25° ± 1°) with a daily lighting schedule of 14 hr of light and 10 hr of darkness, and with free access to Purina rat chow and tap water.

BIOASSAY

The presence of MIS was assayed by a graded organ culture method (9). The urogenital ridge from 14 ½ day female fetuses containing Wolffian and Müllerian ducts were placed on an agar coated stainless steel grid of a Falcon 3010 organ culture dish. Testicular tissue to be assayed, 1–2 mm fragments, were placed adjacent to the ducts. Incubations were performed over wells containing 2 ml of media [(CMRL, 10% fetal calf serum, 200 units penicillin and 200 mcg streptomycin) (Gibco)] at 37° for 72 hr in a humidified atmosphere of 95% air and 5% CO_2. Specimens were then fixed in buffered formalin dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin. The cranial end of the duct was cut in serial crosssections and stained with hematoxylin and eosin. Multiple sections were studied by light microscopy and regression of the duct graded on a scale of 0–5. Five slides with 6–10 sections per slide were presented blindly to 2 independent experienced observers, and the mean grade of their observations was recorded.

LHRH-ANTISERUM (LHRH-AS)

Antiserum to LHRH was generated in rabbits by repeated intradermal/subcutaneous injections of synthetic LHRH conju-

gated to bovine thyroglobulin by bisdiazotized benzidine (Jackson, unpublished data). This technique was similar to that used to generate antibodies to thyrotropin releasing hormone (15). For the initial injection, the complex was emulsified in Freund's complete adjuvant; subsequent injections used incomplete adjuvant. The LHRH antiserum showed no crossreactivity with deamido-LHRH, thyrotropin releasing hormone, somatostatin, vasopressin, triodothyronine, thyroxine, or angiotensin I or II. The antibody reacted with C-terminal nonapeptide, to pentapeptide fragments of LHRH, but not significantly with N-terminal fragments. A 1:280,000 dilution of LHRH antiserum bound 45% of labeled LHRH under the conditions of our radioimmunoassay (Jackson *et al.*, unpublished data).

GONADOTROPINS

FSH was obtained from the NIAMDD, NIH Rat Pituitary Hormone distribution program (NIAMDD Rat FSH-RP-1). The biologic potency obtained from the insert was as follows: FSH, 2.1 × NIH-FSH-S1 by hCG augmentation assay (29); LH, 0.02 × NIH-LH-S1 by ovarian ascorbic acid depletion (OAAD) assay (28); and TSH, 0.3 USP (bovine) units/mg by McKenzie assay (25). The biologic equivalent for NIH-FSH-S1 was 50 IU/mg by hCG augmentation assay (Second International Reference Preparation) (1). Because FSH is crosscontaminated with LH, the following biologic potencies can be expected: FSH-RP-1 has 105 IU FSH and 11.5 IU LH/mg. From Sigma, hCG was obtained, and its biological potency determined with the U.S.P. chorionic gonadotropin reference standard. In order to be certain that the doses of each hormone (20–22) were greater than previously reported for biological replacement, 100 mIU FSH and 5 IU hCG were used. Both solutions were diluted in sterile water and administered in 0.16 ml volume.

The vehicle for the control treatment was sterile water. Intermediate doses of FSH (20 and 60 mI.U.) were given to additional groups of male pups in the same volume.

EXPERIMENTAL PROTOCOL (FIG. 1)

Twenty 13 day old pregnant rats were given 1 ml of LHRH antiserum ip, and another eight rats were treated with an equal volume of normal rabbit serum. On the 20th day of pregnancy, the females were again injected ip with another 1 ml of LHRH antiserum or normal rabbit serum. After delivery, the males were carefully marked as LHRH antiserum or control pups, then were randomly assigned to six other dams who delivered the same day. Each new mother was assigned randomly coded pups from the

PREGNANT MOTHER	PUPS	MIS ASSAY	p VALUES
TREATMENT		n=	
NRS	Water	2.3 ± 0.2 (10)	
LHRH-AS	Water	3.4 ± 0.3 (12)	p<0.01
LHRH-AS	100 mIU FSH	2.3 ± 0.2 (8)	p < 0.01
	60 mIU FSH	2.4 ± 0.6 (5)	N.S.
	20 mIU FSH	2.9 ± 0.6 (6)	N.S.
LHRH-AS	HCG 5 IU	3.4 ± 0.4 (6)	p<0.02

Fig. 2. MIS activity (mean) of testes from 6 day old pups whose mothers were treated with 1 ml LHRH-AS or normal rabbit serum (NRS). Pups from LHRH-AS treated mothers were given: 1) 100 mI.U. FSH, 2) 60 mI.U. FSH, 3) 20 mI.U. FSH, 4) 5 I.U. hCG, and 5) water (postnatal control) daily s.c. for 5 days. Pups from NRS treated mother (pregnancy control) were given an equal volume of water. The means ± SE of the graded organ culture assay are compared.

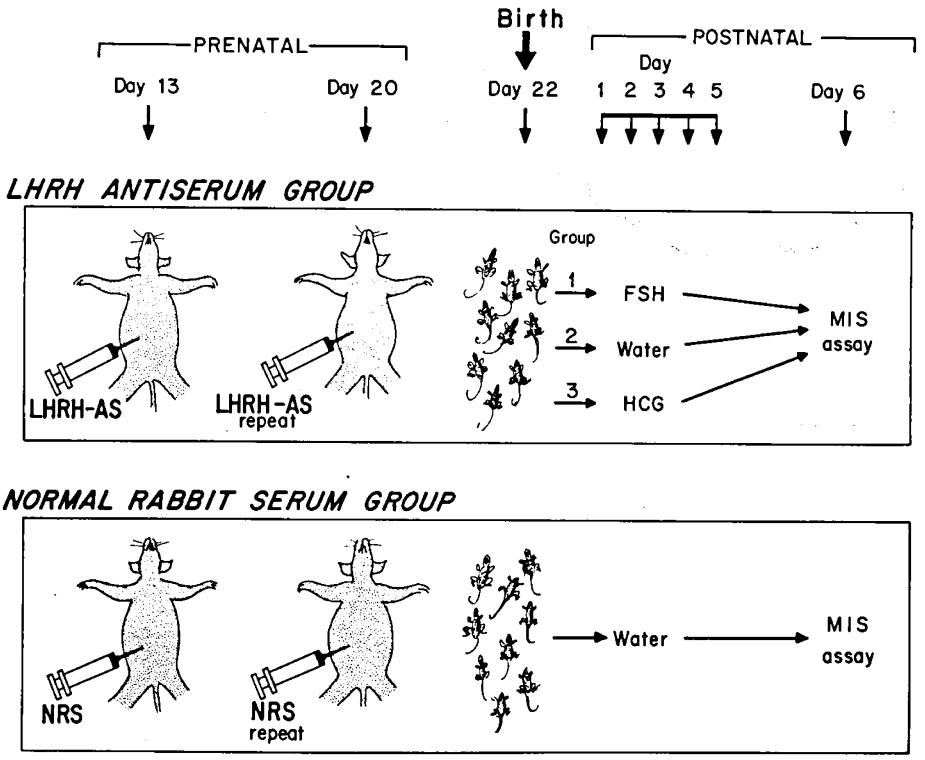

Fig. 1. Schematic representation of experimental protocol. LHRH-AS, 1 ml, or normal rabbit serum (NRS) (pregnancy control) was injected into pregnant dams at gestational ages 13 and 20 days. After birth, pups from LHRH-AS treated mothers received one of the following regimens s.c. daily for 5 days: 1) 100 mI.U. FSH, 2) 60 mI.U. FSH, 3) 20 mI.U. FSH, 4) 5 I.U. hCG, and 5) water (postnatal control); pups from NRS treated mothers received 6) water. Testis from 6-day old pups were assayed for MIS activity.

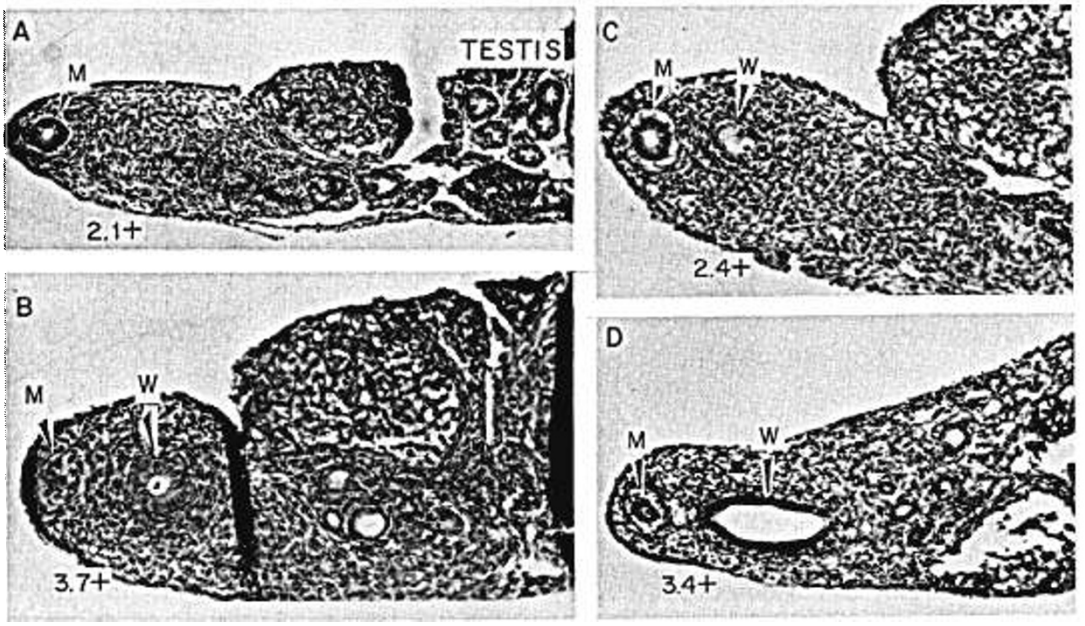

Fig. 3. Müllerian duct (M) regression in the 14½ day female rat embryo urogenital ridge caused by testes from 6 day old male rats after 3 days in organ culture. *A*) Testis of rat born to a dam treated with normal rabbit serum (pregnancy control) and after birth, injected with water for 5 days (2.1 + regression). *B*) Testis of a newborn rat treated daily for 5 days with water born to a mother treated with LHRH-AS (postnatal control) (grade 3.7 + regression). *C*) Pups in this group were given 100 mI.U. FSH after the pregnant dams were injected with LHRH-AS and their testes assayed for regression of Müllerian ducts (2.4 +). *D*) Pups in this group were given 5 I.U. hCG after the mother was injected with LHRH-AS and their testes assayed (3.4 + regression). *(M)* Müllerian duct, *(W)* Wolffian duct.

following groups: pups from LHRH antiserum treated mothers which were subsequently given one of the following treatments, 1) 100 mI.U. FSH; 2) 60 mI.U. FSH; 3) 20 mI.U. FSH; 4) 5 I.U. hCG; and 5) water (postnatal control); pups from mothers treated with normal rabbit serum which were given 6) water (pregnancy control). All injections were given daily s.c. for the first 5 days of postnatal life. Those pups which receive vehicle after LHRH-AS will be defined as postnatal controls and those pups which receive vehicle after normal rabbit serum will be called pregnancy controls.

Tests from pups 6 days of age, were studied for MIS activity because of our previous observation that maternal treatment with LHRH antiserum resulted in the largest increase in MIS secretion relative to controls at this time. The animals were weighed and then decapitated. One testis was removed for MIS assay. Group comparisons were analyzed by unpaired *t* tests. All data are presented as mean ± SE. In addition, the Wilcoxon rank-order test confirmed the statistical significance obtained with the student *t* test.

RESULTS

MÜLLERIAN INHIBITING SUBSTANCE ACTIVITY (FIG. 2 AND 3)

Organ culture from testes of 6-day old pups treated from birth with vehicle showed higher MIS activity (grade 3.4 ± 0.3) if the dam had received LHRH antiserum at 13 and 20 days gestational age as opposed to normal rabbit serum (pregnancy control) (grade 2.3 ± 0.2) ($P < 0.01$). FSH (100 mIU) given after birth to pups from mothers treated with LHRH antiserum reduced MIS secretion (grade 2.3 ± 0.2 *vs.* 3.4 ± 0.3) ($P < 0.01$). These FSH treated pups from LHRH antiserum treated mothers had MIS activity similar to that of control pups from mothers treated with normal rabbit serum (grade 2.3 ± 0.2 *vs.* 2.3 ± 0.2). Pups from mothers treated with LHRH antiserum were given hCG; this failed to alter the increased testicular MIS activity resulting from the LHRH antiserum treatment of their dams (grade 3.4 ± 0.4 *vs.* 3.4 ± 0.3). Body weights in all groups did not differ, inferring that general health was unaltered.

DISCUSSION

The antiserum to LHRH used here has been shown previously to depress serum testosterone transiently in adult rats (3–5). Treatment of pregnant dams with LHRH-AS results in male pups with smaller than normal testes and external genitalia (6). We suggested that the LHRH antibody crossed the maternal-placental barrier and inhibited secretion of the fetal gonadotropins. The present study confirms our previous observation that this apparent inhibition of endogenous gonadotropins results in increased testicular MIS activity (6). Additionally, the present experimental data support our earlier speculation that FSH might inhibit MIS secretion. This was based on the knowledge that FSH controls certain functions of the Sertoli cell (13) and on the evidence that the Sertoli cell is the source of MIS (7, 16). Furthermore, the high serum FSH levels in late fetal and early postnatal life may account for the decreasing MIS during this period. The data clearly indicate that hCG and presumably LH do not restore the inhibitory effect seen with FSH after immunologic blockade with LHRH antiserum. The increasing inhibition of MIS activity with higher doses of FSH suggests a dose response, however the graded inhibition by lower FSH doses, although suggestive, is not statistically significant. This may be due to a small sampling and/or intraassay variation attributable to biologic assays in general. The gonadotropins in the fetal calf serum (14) used in the organ culture MIS assay should not interfere with the results because they are present in both treatment and control groups equally.

This study indicates that MIS secretion may be dependent on hypothalamic function through LHRH regulation of FSH release.

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