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Effect of Vitamin A Deficiency on Luteinizing Hormone Receptors and Adenosine 3′,5′-Monophosphate-Mediated Steroidogenesis in Rat Testicular Tissue (41134)

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Abstract. Weanling male Sprague–Dawley rats were maintained on a vitamin A (retinol)-deficient retinoic acid-supplemented diet for a period of 8 to 14 weeks. After 10 weeks, Leydig cell-enriched preparations from vitamin A-deficient animals had 45% fewer gonadotropin receptors for human luteinizing hormone (hLH). Testicular slices from vitamin A-deficient animals demonstrated decreased formation of adenosine 3′5′-monophosphate (cyclic AMP) and decreased testosterone content in response to hLH stimulation compared to vitamin A-supplemented rats ($P < 0.01$). Our findings suggest that changes which cause hyporesponsiveness in testosterone production from vitamin A-deficient rats can be attributed to a reduction in gonadotropin testicular receptors for hLH and a decreased cyclic AMP production. Testicular morphology was not altered during the first 10 weeks of the experiment, although, after 14 weeks on the experimental diet there was marked degeneration of tubules, cessation of spermatogenesis, and testicular atrophy.

Several workers have demonstrated in recent years that vitamin A (retinol) deficiency in animals results in major lesions in testes, including atrophy and loss of cells of the germinal epithelium (1, 2). Other investigators have reported that retinoic acid in male rats will maintain division and differentiation of epithelial cells but will not support spermatogenesis (2). There have been conflicting reports on the effect of vitamin A deficiency on plasma testosterone levels. Early reports demonstrated no effect of vitamin A deficiency on plasma testosterone (3). A later study reported plasma testosterone and luteinizing hormone (hLH) levels to be decreased in vitamin A deficiency. In the latter case pituitary sections revealed hypertrophied PAS-positive granules with colloid particles in the cytoplasm, as seen in castrated animals (4). Little is known, however, about the effect of vitamin A upon the hormonal role of testes, namely steroidogenesis, and upon its functional coupling to gonadotropin receptors and to adenylate cyclase. These important biochemical characteristics of steroidogenesis in rat testes *in vitro* have already been discussed in previous papers (5–7).

In the present study we have assessed the effect of vitamin A deficiency upon the binding of human luteinizing hormone (hLH) to particulate testicular receptors. We have also assessed the effect of vitamin A deficiency on the relationship between hLH binding, adenosine 3′,5′-monophosphate (cyclic AMP) formation, and testosterone secretion *in vitro*.

We have chosen a method to achieve vitamin A deficiency which did not rely on total vitamin A depletion before institution of retinoic acid as others have done (4, 8). Despite a prolonged waiting period for vitamin A deficiency due to sparing effects by retinoic acid, we were able to obviate some complications of total vitamin A deficiency that could have made the results more difficult to interpret.

Materials and Methods. Male, weanling rats of the Sprague–Dawley variety were housed in a temperature-controlled environment with a 12-hr day, 12-hr night cycle and fed a retinol-deficient, retinoic acid-supplemented diet formulated according to Teklad Test Diets (Madison, Wisc.) as shown in Table I, for periods of 8, 10, and 14 weeks. The rats were sacrificed by in-

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TABLE I. COMPOSITION OF DIETARY REGIMENS

Retinol-deficient diet with added retinoic acid[a]	
Casein, vitamin-free test	180.0
Sucrose	499.9
Corn starch	150.0
Brewer's yeast, powder	80.0
Cottonseed oil	50.0
Mineral mix, USP XIV	40.0
Vitamin D_2, in Corn oil[c]	0.01
All-Trans-Retinoic Acid	0.024
Vitamin A diet[a]	
Casein, vitamin-free test	180.0
Corn starch[b]	649.8
Brewer's yeast, powder	80.0
Cottonseed oil	50.0
Mineral mix, USP XIV	40.0
Vitamin D_2, in corn oil[c]	0.01
Retinyl Palmitate, in corn oil[c]	0.0991

[a] g/kg.

[b] In order to pellet, corn starch must be reduced to 150 g/kg of diet and sucrose added at 499.8 g/kg.

[c] Vitamins supplied the following: (mg/kg corn oil) vitamin D_2, 4.0; retinyl palmitate 2.0.

traperitoneal injection of pentobarbital. Control rats were maintained on identical diets except that retinyl palmitate was substituted for retinoic acid. Two experiments were conducted at different times, but the results are presented together.

Testes were removed, decapsulated, and dropped into 6 ml of cold buffer (0.05 *M* Tris–HCl, pH 7.5, with 0.05 *M* sucrose, 5 m*M* $MgCl_2$, and 0.1% BSA). The tissue was gently disrupted in a glass homogenizer with 30 strokes of a loose-fitting pestle. The homogenate was filtered through two layers of cheese cloth to remove tubules and cellular debris and brought to a final dilution of 1:30 (wt/v) with buffer. Such Leydig cell-enriched preparations (9) were used for hLH binding studies on the same day of preparation.

For cyclic AMP determination decapsulated testes were placed on an ice-cold glass plate and quickly sectioned once longitudinally and three times horizontally taking care to disrupt the tissue as little as possible. The tissue was placed in Krebs–Heinsleit buffer, gassed with 95% O_2/5% CO_2, and placed in a shaking water bath at 34° and 120 cycles per min. Tubes were placed at a 30-degree angle, leaning in the direction of shaking, for 40 min. Five micrograms of hLH was then added to appropriate tubes. All tubes had 0.1 m*M* 1-methyl-3-isobutylxanthine (MIX), a potent inhibitor of phosphodiesterase activity, added. Tubes were again gassed and reincubated at 34° for 2 hr after which TCA was added to each tube to a final concentration of 5%. [^{3}H]Cyclic AMP (3000 cpm/ml, sp act 42.5 Ci/mmole, New England Nuclear, Boston, Mass.) was added to each tube to measure recoveries in the determination of cyclic AMP. The contents of the tubes were mixed, sonified, and centrifuged at 2000 *g* for 25 min at 4°. The supernate was removed and cyclic AMP was isolated using Dowex 50W × 4 ion-exchange columns. One milliliter of the supernate was placed on the 5-cm column followed by 2 ml of 5% TCA, then 6 ml H_2O, and a final 6 ml of H_2O, and the effluents combined and lyophilized for cyclic AMP determination. For each 1 ml of specimen applied to the column, 320 μl of 0.05 *M* acetate buffer, pH 4.0, was added to reconstitute. Another aliquot of the supernate (vide supra) was lyophilized for testosterone determination. The pellets were dissolved in NaOH and protein was determined according to Lowry *et al.* (10).

Cyclic AMP was determined by a modification of the competitive protein binding method of Gilman (11). The original volume of the sample (20 μl) in acetate buffer (vide supra) was added in duplicate to tubes with 15 μl [^{3}H]cyclic AMP (about 30,000 cpm), and 15 μl protein kinase diluted 1:6 with aqueous 1 mg/ml BSA. Rabbit protein kinase was a generous gift from Dr. Gilbert Rinard, Emory University. Standards of 0.5 to 20.0 pmole in 20 μl acetate buffer were treated as the samples. Tubes were incubated at 4° and at the end of 1 hr of incubation, 5 ml cold 0.05 *M* phosphate buffer, pH 6.2, was added to tubes. Millipore Type HA filters were wet with 3 ml of pH 6.2 buffer, then the sample was added in a volume of 5 ml buffer in the presence of a vacuum. The filters containing enzyme bound cyclic AMP were allowed to dissolve in scintillation cocktail and their radioactivity determined.

hLH-binding to Leydig cell membranes was determined by the method of Reichert

et al. (9). LER-960 hLH was iodinated by a modification of the chloramine-T method. Specific activity was equal to the number of microcuries added times percentage iodination, divided by the number of micrograms of hormone added. Nonspecific binding was that amount of labeled hormone which was bound in the presence of 100-fold excess of unlabeled hormone.

Testosterone was determined by radioimmunoassay (RIA) on an aliquot of the incubation mixture. [1,2,6,7,16,17-^{3}H]Testosterone (sp act, 152 Ci/mmole) was purchased from New England Nuclear and checked for purity by thin-layer chromatography. Purified crystalline testosterone standard was obtained from Sigma Chemical Company (St. Louis, Mo.). RIA for testosterone was done, after ether extraction, evaporation, and reconstitution in ethanol using the method of Ismail *et al.* (12). Anti-testosterone antibody (to testosterone-3-carboxymethyloxime BSA) was purchased from Wien Laboratories, Succasunna, New Jersey. The minimum detectable dose varied from 12 to 20 pg (mean 16) and the intraassay and interassay coefficients of variance (at a 2 ng/ml concentration) were 6 and 6.8%, respectively. The antibody is highly specific reacting only with 5-androstan-17β-ol-3-one (dihydrotestosterone) by 13%.

Vitamin A was determined by a modification of the method of Drujan *et al.* (13). A piece of tissue was ground with four times its weight of anhydrous sodium sulfate, transferred to a glass-stoppered centrifuge tube, and saponified for 10 min at 75° in a nitrogen atmosphere with alcoholic KOH (ethyl alcohol–60% solution of KOH in water, 5:1, v/v). After saponification, 10 ml of water was added and vitamin A was extracted three times with 10 ml ether. The ether was evaporated to dryness and the residue dissolved in 1 ml petroleum ether, applied to alumina columns 7 cm in Pasteur pipets, and eluted with petroleum ether, 6 and 50% diethyl ether in petroleum ether in that order. The eluates from the 6 and 50% diethyl ether in petroleum ether washes were combined and evaporated under nitrogen. The residue was dissolved in a minimum amount of chloroform and brought to 10 ml with *n*-butanol and the fluorescence intensity measured using a 360-nm excitation filter and 490-nm emission filter.

Cells were evaluated by histochemical staining for 3β-hydroxysteroid dehydrogenase-Δ^5,Δ^4-isomerase complex according to the method of Mendelson *et al.* (14). Sections of testes from experimental and control animals were stained with hematoxylin and eosin and evaluated for microscopic changes.

All RIA results were analyzed by the computerized method of Rodbard and Lewald (15). Affinity constants and binding capacities of gonadotropin receptors were determined by regression analysis of Scatchard plots (16). Regression analyses were performed by the method of least squares. Student's *t* test was used to test for significant differences between the measured parameters. All means are shown ± SD unless otherwise noted. We consider any *P* value less than 0.05 to indicate statistical significance.

Results. Of 21 rats maintained on vitamin A-deficient diets, one died of unknown reasons and 10 became vitamin A-deficient by the criterion set for this study; namely, less than 10 μg of vitamin A per gram wet liver weight.

In results not shown here, when rats were sacrificed after 8 weeks on the experimental diet, there was no apparent difference between hormone binding to Leydig cells in the experimental and in the control animals; the values for maximum binding being 6.4 ± 1.1 and 7.05 ± 1.2%, respectively. Scatchard plot analysis of binding data derived from Leydig cell-enriched preparations of control animals revealed the presence of a single class of binding sites with relatively high affinity for ^{125}I-hLH. The affinity constant as determined by Scatchard analysis was almost identical with that obtained by direct analysis of saturation curve data, the values being 1.09 ± 10^{10} and 1.21 × 10^{10} M^{-1}, respectively. The binding capacity for ^{125}I-hLH was about 1.0 × 10^{-12} mole/g wet tissue by both methods. Hill plots of the hLH binding data showed a slope of 1.0, suggesting the presence of a single class of gonadotropin receptor sites with no interac-

tion between themselves. No dissociation of bound hormone from the membrane receptor sites was detectable after incubation of ^{125}I-hLH-labeled receptors for 2 hr at 26°, and only 10% reduction of binding was observed after incubation at 26° for 12 hr. Apparent affinity constants obtained in this study were similar to those reported by Catt *et al.* (17) and by Ketelslegers *et al.* (18) the values being 2.7×10^{10} and $1.0 \times 10^{10}\ M^{-1}$, respectively. Scatchard plot analysis of binding data derived from testicular preparations from seven animals, after 8 weeks on the experimental diet, gave the following values: $0.813 \pm 0.275 \times 10^{10}\ M^{-1}$ and 0.745×10^{-12} mole/g wet tissue, for affinity constant and binding capacity, respectively. These values did not significantly differ from those reported for control animals. Meanwhile, hepatic vitamin A stores were not different between experimental and control animals at 8 weeks of feeding, the values being 31.4 ± 13.5 and 32.1 ± 18.0 μg/g wet weight of liver, respectively.

After 10 weeks on the experimental diet, 11 of 15 rats had hepatic vitamin A concentrations of 10 μg/g wet weight of liver or less (Table II, mean value 5.8 ± 3.3) whereas control rats had 32.1 ± 15.6 μg/g wet weight of liver. Table II gives values of maximum binding of ^{125}I-hLH to Leydig cell-enriched preparations, cyclic AMP concentrations, and testosterone concentrations between experimental and control animals. Differences in maximum binding were significant ($P < 0.005$), the values being 3.39 ± 1.19 and 5.77 ± 1.5% for vitamin A deficient and control rats, respectively. Also significant ($P < 0.005$) were cyclic AMP and testosterone concentrations between vitamin A-deficient and control rats; the values for cyclic AMP being 2.19 ± 2.7 pmole/mg protein and 15.8 ± 8.0 pmole/mg protein, respectively. For testosterone, values were 1.31 ± 1.7 ng/mg protein and 3.14 ± 1.37 ng/mg protein for vitamin A-deficient and control rats, respectively.

Several animals failed to become vitamin A deficient, i.e., hepatic vitamin A stores

TABLE II. LH BINDING, CYCLIC AMP, AND TESTOSTERONE LEVELS IN RATS FED EXPERIMENTAL AND CONTROL DIETS FOR 10 WEEKS AFTER WEANING

Pair[a]	Animal	Binding[b] (%)	cyclic AMP (pmole/mg protein)	Testosterone (ng/mg protein)	Vitamin A (g/g wet weight liver)
1	Control	7.3	23.4	5.30	27.7
	Experimental	3.2	4.1	3.69	0.4
2	Control	7.3	13.6	5.87	68.4
	Experimental	3.7	4.4	3.44	6.5
3	Control	5.9	11.2	3.91	29.1
	Experimental	4.3	0.1	1.00	3.0
4	Control	7.3	4.9	2.28	12.8
	Experimental	4.1	0.9	0.80	9.7
5	Control	3.4	5.15	*[c]	41.1
	Experimental	1.1	1.10	3.83	3.9
6	Control	3.8	16.95	4.13	26.5
	Experimental	1.6	0.6	0.60	6.4
7	Control	9.2	13.2	2.40	14.9
	Experimental	7.1	5.0	0.18	9.6
8	Control	5.5	20.5	1.08	34.4
	Experimental	3.7	0.7	0.15	10.9
9	Control	5.5	12.3	1.29	26.2
	Experimental	2.3	0.6	0.20	5.1
10	Control	4.3	30.4	3.28	48.2
	Experimental	2.2	1.8	0.10	3.0
11	Control	4.4	19.5	1.84	23.0
	Experimental	4.0	9.1	1.10	6.3

[a] Pairs represent animals sacrificed on the same day.
[b] Expressed as bound/total ^{125}I-hLH added in the maximum binding tube. Nonspecific binding was subtracted.
[c] Sample lost.

<10.0 μg/g wet weight, and they were excluded from calculations as experimental or controls. Their vitamin A status did not qualify them as vitamin A deficient, and their vitamin A-deficient diet excluded them from controls. It was felt that use of these animals would introduce uncontrolled variables because they fell into neither control nor test groups. Table III shows the binding constants of hLH to testicular receptors calculated by Scatchard plot analysis of binding data determined in experimental and control animals. Four pairs of animals gave Leydig cell-enriched preparations which upon addition of increasing amounts of hLH in the presence of a fixed amount of ^{125}I-hLH failed to inhibit binding of the labeled LH in a dose-dependent fashion so that no Scatchard plot could be drawn, and no values were obtained for binding sites or affinity constants. The alternative to obtaining no value for affinity constants or binding sites would have been to take 0 as a value in these cases. It was felt that the more conservative approach would be to use only preparations from which values could be calculated, even though using those preparations showing no binding would have supported the premise that vitamin A deficiency affects high-affinity low-capacity binding sites for LH. Otherwise, differences in concentration of binding sites between experimental and control animals were significant ($P < 0.005$); the mean values being 0.445 ± 0.10 and 0.76 ± 0.15 × 10^{-12} mol/mg protein, respectively. Significant ($P < 0.05$) difference between experimental and control animals was not proven as far as affinity constants was concerned.

Mean total body weights did not differ between experimental and control animals, through the 10th week. At the 14th week, however, the experimental animals reached a weight plateau and there was a significant ($P < 0.005$) difference in testicular weights between 9 experimental and 9 control animals, the values being 0.788 ± 0.85 and 1.63 ± 0.84 g, respectively. These results indicated testicular atrophy of the experimental animals.

There were an average of 5.52 ± 1.12 × 10^6 cells/ml homogenate, including sperm, which made up approximately 17 ± 4% of the total cells. The presence of sperm indicated some contamination with other cells of the germinal epithelium. Of the cells counted, 39.8 ± 5% of control cells and 39.5 ± 12% of test cells appeared to have 3β-hydroxysteroid dehydrogenase-Δ^5-Δ^4-isomerase complex activity, as evidenced by dark red granules in the cell. Histological differences between experimental and control animals were not observed during the 10-week experimental period. They did become apparent, however, at the 14th

TABLE III. LH BINDING PARAMETERS FOR RETINOL-DEFICIENT AND CONTROL ANIMALS AFTER 10 WEEKS

	Binding sites[b]		Affinity constant[c]	
Pair[a]	Experimental	Control	Experimental	Control
1	0.007	0.013	1.714	0.419
2	*[d]	0.014	*	1.600
3	0.006	0.020	0.288	0.347
4	0.010	0.013	0.975	1.297
5	*	0.007	*	0.472
6	*	0.010	*	0.536
7	0.008	0.011	0.800	0.500
8	0.008	0.010	0.800	0.642
9	*	0.012	*	0.510
10	0.011	0.030	0.300	0.720
11	0.009	0.021	0.400	0.750

[a] Pairs represent animals sacrificed on the same day.

[b] pmole/g wet tissue. Mean values 0.0084 ± 0.017 and 0.0147 ± 0.0066 for experimental and control, respectively. $P < 0.005$.

[c] $\times 10^{10}$ M^{-1}. Mean values 0.754 ± 0.5 and 0.708 ± 0.39 for experimental and control, respectively.

[d] Failed to show reversible binding to ^{125}I-hLH.

week. Staining with hematoxylin showed marked degeneration of tubules and cessation of spermatogenesis.

Discussion. The results clearly show that at a time when vitamin A stores were depleted in 11 out of 21 animals which we maintained on a retinol-deficient diet, the experimental animals demonstrated a small but significant decrease in the number of hLH receptor binding sites. No change in affinity constant was seen, suggesting no alterations in the quality of LH receptors as a result of vitamin A deficiency. Our results also show a significant reduction in the concentration of cyclic AMP and of testosterone in Leydig cell-enriched preparations from vitamin A-deficient animals. These findings seem to suggest that decreased steroidogenesis in Leydig cells from vitamin A-deficient rats is probably attributable to a reduction in cyclic AMP production in association with a reduction in hLH receptors. That cyclic AMP formation and steroidogenesis are functionally coupled to hLH binding to receptors for gonadotropins in Leydig cells has already been discussed in previous publications (17, 18) and in the introduction of this paper.

On the other hand, recent work has repeatedly shown that retinol acts as a glycosylating agent in glycoprotein synthesis (19, 20). Since other studies have already shown that the hLH receptor is a glycoprotein (17, 18) we may assume that in vitamin A deficiency these receptors may not be completely glycosylated. Recently, more data have been accumulated on the effects of incomplete glycosylation of the protein of glycopeptide hormones which bind to membrane receptors (19). The distinction between alterations in the receptor glycoprotein and ligand glycoprotein is important, but the possible similarities in effects of incomplete glycosylation must not be overlooked. It is of interest to note here, that Catt *et al.* (17) have shown that changes in the carbohydrate side chains of glycoprotein hormones have a profound effect on the binding of hLH and of human chorionic gonadotropin (hCG) to testicular receptors and in the ensuing biological activity of the hormones. Addition or removal of galactose residues, for instance, results in a decrease or a return to the original level of binding activity. The reduction in Leydig cell stimulation by asialo-hCG and asialogalacto-hCG has also been shown (21). The ability of the modified glycoprotein to stimulate cyclic AMP formation in the testes is also reduced and that was shown by several workers (17, 18, 22).

Little is known, however, about changes in carbohydrate composition of the testicular receptors and the effect on the binding of hLH and hCG to those receptors. It is possible that defects in the glycosylation of testicular receptors could result in decreased synthesis and availability of those receptors, and in decreased binding of hLH which causes hyporesponsiveness to cyclic AMP and to testosterone production, as the present study seems to suggest. It would, therefore, be of considerable importance to investigate the role of vitamin A in the glycosylation process of rat testicular receptors and the effect that alterations in carbohydrate composition of testicular receptors have on the binding of hLH.

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