THE AGING LEYDIG CELL:

2. TWO DISTINCT POPULATIONS OF LEYDIG CELLS AND THE POSSIBLE SITE OF DEFECTIVE STEROIDOGENESIS

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ABSTRACT

Using metrizamide gradient centrifugation two populations of Leydig cells were found in both 60-90 day-old and 24 month-old rats. Cells from both Band 2 (B_2) and Band 3 (B_3) responded to LH stimulation with increased cyclic AMP formation; however, only B_3 cells produced significant amounts of testosterone. Cells from both B_2 and B_3 of the old rats synthesized less cyclic AMP and testosterone than cells from their younger counterparts. In response to LH stimulation, 0.01 - 1.0 mIU/ml, no appreciable difference of cyclic AMP formation could be detected between young and old Leydig cells. Maximal testosterone production occurred when 1 mIU/ml LH was used. Only when LH concentration was increased to 10 and 100 mIU/ml, did young Leydig cells produce significantly more cyclic AMP than old Leydig cells. After addition of $5x10^{-7}M$ of pregnenolone or progesterone to the incubation medium, both young and old Leydig cells produced comparable amounts of testosterone. These results demonstrate no impairment of old rat Leydig cells to synthesize testosterone from pregnenolone and progesterone.

INTRODUCTION

Plasma testosterone concentrations in male mammals decrease markedly during aging (1-4). Several mechanisms could be operative - hypothalamic dysfunction, decreased luteinizing hormone (LH) release, end-organ defects (Leydig cells) or combinations of these. In Sprague-Dawley rats, we have previously found that plasma testosterone levels were significantly lower in 24-month old males than 60-90 day-old animals both before and after a single injection of human chorionic gonadotropin (5). Even following three weeks of hCG administration circulating testosterone levels of old

rats did not reach basal concentrations present in young animals (5). Furthermore, in response to LH and 8-bromo-adenosine 3',5'-monophosphate (8-bromo-cyclic AMP) in vitro stimulation, purified young Leydig cells produced significantly more testosterone than Leydig cells from old rats (5). These results suggested defects in old Leydig cells causing reduced testosterone response with the major defects beyond cyclic AMP formation.

Recently, Payne et al. (6) reported the existence of two populations of Leydig cells from interstitial tissue of mature rats. The binding affinities and the concentration of LH-binding sites per Leydig cell were similar in both populations. However, only population II (Band 3, B_3), responded to hCG stimulation with increased testosterone formation. It was suggested that population I (Band 2, B_2), which did not respond, represented immature Leydig cells. The effects of aging on these two populations of cells remains unknown. Present studies were designed to further investigate the defects in steroidogenesis of old Leydig cells, and the effects of aging on the two populations of Leydig cells.

MATERIALS AND METHODS

1. Materials:

Testosterone, 3β-hydroxy-5-pregnen-20-one (pregnenolone), and 4-pregnene-3,20-dione (progesterone) (Steraloids, Wilton, New Hampshire) were recrystalized prior to use. {1,2,6,7,^{3}H}-testosterone (100 Ci/mmole) and cyclic AMP-2'-O-succinyl-N-(^{125}I)-iodotyrosine methyl ester (150 Ci/mmole) were obtained from New England Nuclear, Boston, Massachusetts. Other materials included: metrizamide (centrifugation grade) - Accurate Chemicals, Hicksville, New York; collagenase (Type I), 1-methyl-3-isobutyl xanthine (MIX) and bovine serum albumin (BSA) - Sigma, St. Louis, Missouri; medium 199 with bicarbonate buffer - Grand Island Biological Company, Grand Island, New York; and human pituitary luteinizing hormone, LH A-3 (LER-1549) made available by the National Pituitary Agency, has 2,225 IU/mg of LH and 1.2 IU/mg of FSH by bioassay.

2. Animals:

Sprague-Dawley rats, ranging from 60-90 days or 24 months of age were obtained from Zivic-Miller Laboratories, Allison Park, Pennsylvania. Only healthy animals were used.

3. In Vitro Studies:

There were marked variations in the amounts of cyclic AMP and testosterone produced by different batches of cell preparations. This may be related in part to cell processing. Therefore, in each experiment testes from one young and one old rat were collected and processed simultaneously. Interstitial cells were obtained by collagenase dispersion of testes as described previously (5). The crude interstitial cell pellet (approximately 10^8 interstitial cells) was resuspended in 1 ml Medium 199-BSA and applied to a 20 ml 0-32% continuous Metrizamide density gradient prepared by an ISCO gradient former (Model 570, Instrumentation Specialties Co.). Centrifugation of each gradient at 3,000xg for 10 minutes resulted in the appearance of 5 bands of cells. Cells from each band were removed by aspiration, washed twice with Medium 199-BSA, collected by centrifugation, and then resuspended in Medium 199-BSA with 0.1 mM MIX. Cells from the second and the third band were used for the present studies. Cell density was determined using a hemocytometer. The percentage of purified cells in the third band staining positively for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was over 90%.

Equal numbers of cells from the young and old rats were used in each incubation (10^5 cells/ml). Purified cells were incubated in duplicate for 3h in a Dubnoff Shaking Incubator at 34°C under 95% O_2/5% CO_2 in Medium 199-BSA-MIX with various concentrations of LH, pregnenolone ($5x10^{-7}$M) or progesterone ($5x10^{-7}$M). At the end of incubation, medium was centrifuged immediately at 3,000xg and the supernatant was stored at -20°C until assayed for testosterone and cyclic AMP.

4. Testosterone Radioimmunoassay:

Testosterone was measured by radioimmunoassay on ether-extracted aliquots of incubation medium without prior chromatography as described previously (7). Because of highly specific testosterone antiserum, it was not necessary to chromatograph the extracted samples to separate pregnenolone and progesterone from testosterone. The addition of 10^{-6}M pregnenolone or progesterone separately to medium controls without cells gave apparent "testosterone" values of only 1% and 2%, respectively, of pregnenolone-only and progesterone-only stimulated testosterone levels of interstitial cells. Therefore, the values reported herein were not adjusted for this minor cross reactivity.

5. Cyclic AMP Radioimmunoassay:

Cyclic AMP assay was performed as previously reported (7,8). The procedure of Steiner et al. (9) was used with the addition of the succinylation step (10). Each specimen was analyzed in duplicate and all samples from a single study were determined together. All results are expressed per 10^6 cells.

Statistical analysis of the data were carried out using paired or non-paired Student's t tests.

RESULTS

1. Effects of Aging on Two Populations of Leydig Cells:

The cyclic AMP responses to LH stimulation of Leydig cells from the second and third bands (B_2 and B_3 respectively) are presented in Fig. 1. Cells from both B_2 and B_3 of the young rats responded to LH with increased

cyclic AMP formation. B_2 cells increased cyclic AMP synthesis from a control of 12.3 ± 2.1 pmoles (mean ± SE) to 15.6 ± 1.7, 29.4 ± 3.5 and 50.8 ± 7.3 with the addition of 1, 10, and 100 mIU/ml of LH, respectively. The cells from the third band (B_3) increased cyclic AMP formation from 11.3 ± 3.2 to 190.7 ± 53.9 pmoles in response to 100 mIU/ml LH, which was 3.7-fold higher than the B_2 cells.

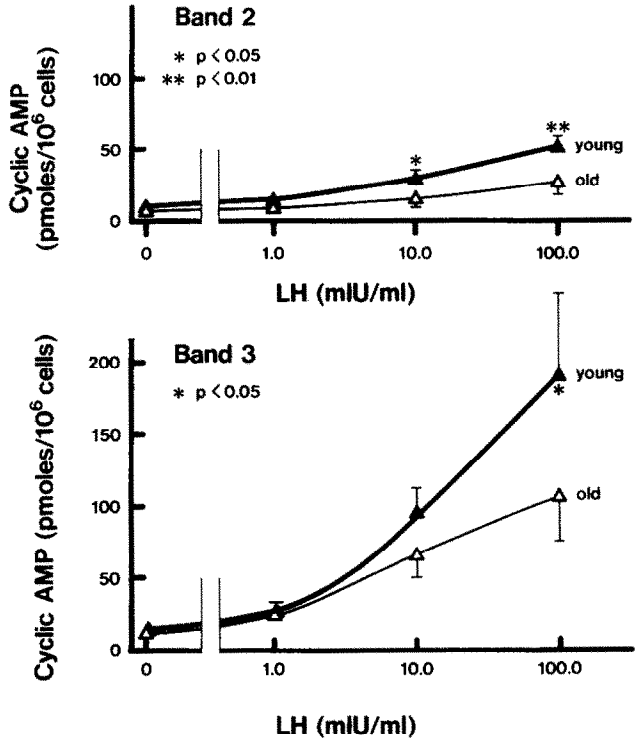

Fig. 1: Dose-response curves of purified Leydig cells from the second (upper panel) and the third band (lower panel) of young and old rats to LH stimulation. Results are the mean ± SE of five experiments.

Both B_2 and B_3 cells of old rats also produced significantly increased amounts of cyclic AMP with the addition of LH 10 and 100 mIU/ml. However, cyclic AMP produced by old Leydig cells was significantly lower than that of the young Leydig cells (Fig. 1).

Although both populations of cells (B_2 and B_3) responded to LH stimulation with increases in cyclic AMP formation, only B_3 cells had the capacity to synthesize significant amounts of testosterone. Maximal testosterone production occurred when 1 mIU/ml LH was used and no significant increases in testosterone formation with higher doses of LH ($p>0.05$). Testosterone formation of the young and old Leydig cells from the second band were negligible when compared to the third band (Table 1).

TABLE 1: TESTOSTERONE RESPONSES OF BAND 2 AND BAND 3 CELLS TO LH STIMULATION

Testosterone ng/10^6 cells

	Band 2		Band 3	
	Young	Old	Young	Old
Control	0.10 ± 0.07	0.02 ± 0.02	5.5 ± 2.1	10.8 ± 5.8
LH 1.0 mIU/ml	0.27 ± 0.10	0.04 ± 0.03	55.6 ± 11.5	30.8 ± 12.1
LH 10 mIU/ml	0.22 ± 0.07	0.04 ± 0.03	53.5 ± 17.6	34.0 ± 10.5
LH 100 mIU/ml	0.25 ± 0.01	0.06 ± 0.03	87.6 ± 17.6	34.1 ± 11.4

B_2 and B_3 cells were incubated with increasing amount of LH and testosterone levels were measured after 3h incubations. Results are the mean ± SE of five experiments.

2. In Vitro Response of Purified Leydig Cells to LH Stimulation:

To further investigate the dose-relationships between LH, testosterone and cyclic AMP, purified Leydig cells from the third band were incubated with increasing amounts of LH. Testosterone and cyclic AMP were measured after 3h incubations. The results are depicted in Fig. 2.

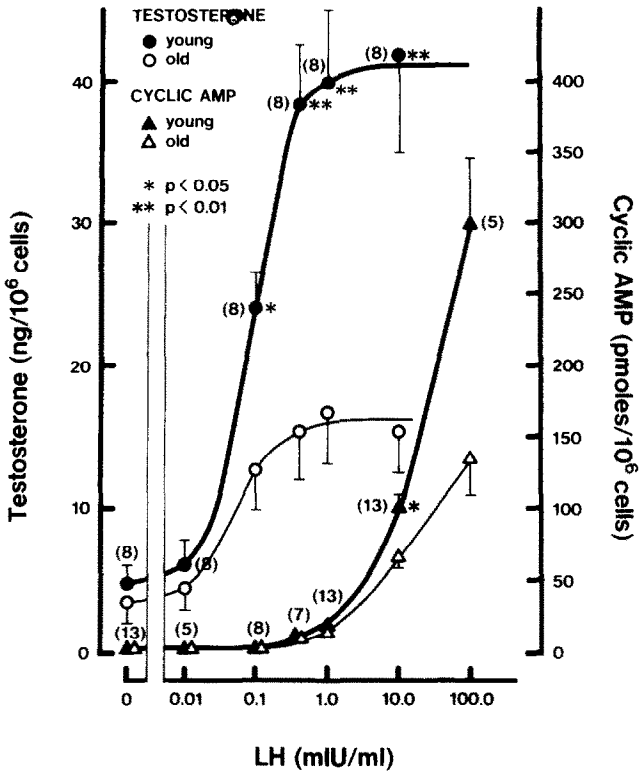

Fig. 2: Dose-response curves of purified Leydig cells of young and old rats to LH stimulation. Numbers in the parentheses indicate the number of pairs of experiments. Results are the mean ± SE. *p<0.05, **p<0.01 as compared to old rats with the same concentrations of added LH.

Baseline cyclic AMP levels in the incubation medium of the young and old Leydig cells were 2.22 ± 0.58 pmoles and 3.20 ± 0.80 pmoles, respectively, which were not significantly different from each other. In response to LH stimulation, no appreciable changes of cyclic AMP levels could be detected with 0.01 and 0.1 mIU/ml of LH. After adding 0.5 mIU/ml of LH to the incubation medium, young Leydig cells produced 8.28 ± 2.38 pmoles/ 10^6 cells of cyclic AMP, comparable to that released by the old Leydig

cells, 9.76 ± 2.06 pmoles. At this concentration of LH, testosterone synthesis was maximal for both young and old groups. As shown in Fig. 2, Leydig cells from the young rats produced significantly more testosterone as compared with cells from the old rats. However, young Leydig cells produced significantly higher amounts of cyclic AMP only when LH concentration reached 10 mIU/ml.

3. Effects of Aging on Conversion of Pregnenolone and Progesterone to Testosterone:

Purified Leydig cells from the third band were incubated with $5x10^{-7}$M pregnenolone or progesterone, and testosterone concentrations were measured after 3h incubations. With the addition of $5x10^{-7}$M pregnenolone, young Leydig cells produced 91.85 ± 21.53 ng of testosterone (n=12), which is not significantly different from that of the old Leydig cells, 71.62 ± 14.71 ng (n=12; $p>0.05$).

In response to $5x10^{-7}$M progesterone, Leydig cells from the young rats produced 133.2 ± 37.4 ng of testosterone (n=12), which were similar to that of Leydig cells from old rats, 103.4 ± 20.6 ng (n=12; $p>0.05$).

These results suggest that the capacity of Leydig cells from the old rats to synthesize testosterone from pregnenolone and progesterone is not impaired.

DISCUSSION

Present results confirm the presence of two populations of Leydig cells. Cells from both B_2 and B_3 responded to LH stimulation with increased cyclic AMP formation; however, only B_3 cells produced significant amounts of testosterone. Payne et al. (6) postulated that Leydig cells from population I (B_2 in this study) were immature cells which have not attained the capacity to respond to gonadotropin. However, in the present study, Leydig

cells from 24-month old rats still exhibit two distinct populations. Cells in both age groups have intact hCG-LH receptor-adenylate cyclase systems, while only B_3 cells have the ability to synthesize testosterone. Whether "B_2" cells represent "immature" cells which have never attained the capacity to synthesize androgen or represent "deteriorated" old cells which are no longer able to form androgen remains unclear. However, our study suggests that both B_2 and B_3 cells from old rats synthesize significantly less cyclic AMP and testosterone respectively than younger animals.

Several mechanisms appear responsible for altered testicular function in aging rats. Reduced LH-releasing hormone in the hypothalamus, decreased LH content in the pituitary gland and lower plasma LH levels have all been reported in old rats as compared to young ones (11, 12). Our previous report (5) and the present studies suggest that there are intrinsic defects in Leydig cell function. Present studies show that testosterone formation by Leydig cells from the old rats was markedly reduced when compared with young rats. Production of testosterone plateaued with added LH using $\geq$ 1 mIU/ml in both groups, while cyclic AMP responses to LH were comparable at both ages until LH was increased to over 10 mIU/ml. Thus, the major defects of testosterone formation are beyond cyclic AMP formation. Furthermore, in this study, we demonstrate that the conversion of pregnenolone and progesterone to testosterone remains intact in the old Leydig cells. Our results differ from that of Chan *et al.* who incubated testes from 4 and 18 month-old rats with labeled progesterone (13). In the older rats, testosterone production was significantly lower. They used Long Evans rats, while Sprague-Dawley rats were used for the present studies and strain differences may account for disparity of results.

It is generally agreed that stimulation of steroid synthesis by the tropic hormone, LH, results from an increase in the rate of conversion of cholesterol to pregnenolone in mitochondria of Leydig cells (14). More recent studies suggest that this stimulation may, in turn, result from enhanced transport of cholesterol to the side chain cleavage enzyme in mitochondria involving intracellular actin (15). Our present results suggest that either of these two steps in the LH-stimulated biosynthesis of testosterone may be responsible for defective steroidogenesis in old Leydig cells since the conversion of pregnenolone and progesterone to testosterone, which take place on the smooth endoplasmic reticulum, were intact in the old Sprague-Dawley rats.

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