SIGNIFICANCE OF THE Δ^5 AND Δ^4 STEROIDOGENIC PATHWAYS IN THE HAMSTER PREOVULATORY FOLLICLE

Anastasia Makris, Dian Olsen and Kenneth J. Ryan
Laboratory of Human Reproduction and Reproductive Biology
Harvard Medical School
Boston, Massachusetts 02115

Received 12-19-83

ABSTRACT

Isolated hamster granulosa cells and theca from preovulatory follicles were incubated in vitro for 2 and 6 h in the absence/or presence of LH and steroid substrates. The purpose of the experiments was to determine, in theca, the relative activities of the Δ^5 and Δ^4 pathways under controlled conditions, and to compare the ability of granulosa cells and theca to form progesterone from exogenous pregnenolone. The results of the experiments show that the Δ^5pathway in theca predominates before and up to 2 h after LH stimulation. The delayed effect of LH after 2 h is a switch from Δ^5 to Δ^4 as the major metabolic pathway. Progesterone formation from exogenous pregnenolone is 7 to 10 times greater in unstimulated granulosa cells than in theca. Acute effects of LH lead to increased conversion of exogenous pregnenolone to progesterone in granulosa cells but not theca. LH does, however, acutely stimulate the thecal conversion of DHEA to androstenedione. The longer term effect of LH in both cell types is to increase pregnenolone conversion to progesterone.

INTRODUCTION

We have previously reported that in vitro progesterone (4-pregnene-3,20-dione) synthesis by the hamster follicular tissue has the following characteristics. 1) LH- or cAMP-stimulated acute progesterone synthesis (within 15 min) is seen only in the intact or recombined follicle (1). 2) progesterone synthesis by LH-stimulated isolated granulosa cells or theca is not measurable by RIA until 4-6 h after initial LH stimulation (1). In addition, progesterone synthesis by the intact follicle appears biphasic (2,3). In 10 h time course studies the first phase of LH stimulated acute progesterone synthesis (0-5 h) is not sensitive to either puromycin or cycloheximide inhibition. The inhibitors, however, prevent accumulation of progesterone after 5 h. The temporal characteristics of progesterone synthesis in these studies are parallel to the acute follicular response

to LH and the delayed progesterone synthesis seen when isolated granulosa cells or theca are stimulated with LH (1). These observations are dependent on using preovulatory follicle tissue isolated just prior to the preovulatory gonadotropin surge (4).

The characteristics of hamster follicle progesterone synthesis implies a synergistic response between theca and granulosa cells to LH resulting in acute progesterone synthesis. The reported observations of various investigators concerning steroidogenic pathways and the respective enzyme systems in ovarian, and, in particular follicular tissue (5-14) have led us to test the theory that the synergism is a result of thecal pregnenolone (3ß-hydroxy-5-pregnen-20-one) conversion to progesterone by granulosa cells. The data presented in this report are derived from experiments designed to test the following ideas: 1) prior to and immediately after the LH surge (or in vitro LH stimulation), the theca have a more active Δ^5 than Δ^4 steroidogenic pathway; 2) LH stimulates an acute synthesis of pregnenolone which is metabolized to androgens via the Δ^5 pathway; 3) granulosa cells before and for up to 4-6 h after the LH surge are, on a per follicle basis, more efficient than theca in converting pregnenolone to progesterone and finally 4) the partial longer term effect of LH is to direct thecal steroidogenesis from a Δ^5 to a Δ^4 pathway.

MATERIALS AND METHODS

Adult LAK:LVG (SYR) hamsters (Lakeview Hamster Colony) were sacrificed at proestrus prior to the preovulatory LH surge (4). Reproductive cycles were established by vaginal smears (5). Preovulatory follicles were dissected (average 8/pair of ovaries), cleaned and pooled. The follicles were used intact or dissected as previously described (1) to obtain isolated theca and granulosa cells. Each incubation vessel in all the experiments contained either 1 follicle, theca from 1 follicle, or granulosa cells from 1 follicle (approximately 50,000 cells).

The tissue was incubated in 12 x 75 mm tubes containing 1 ml of McCoy's medium (Grand Island Biological), 1% w:v BSA (Sigma). The tubes were placed in a 37°C Dubnoff water bath operating at 60 cycles/min. The incubation time varied with the experiment.

Specific experiments called for addition to the incubation medium of LH and/or radioactive or non-radioactive steroids. The LH was human LH LER (960) kindly donated by Dr. Leo Reichert, National Institutes of Health. Non-radioactive steroids used in the incubations were pregnenolone and DHEA (3ß-hydroxy-5- -androsten-17-one). They were obtained from Sigma Chemical and purified prior to use by celite chromatography. Pregnenolone [7-^{3}H(N)] (10-25 Ci/mmol) was obtained from New England Nuclear, purified prior to use by celite chromatography and diluted with non-radioactive pregnenolone to specific activities that were dependent on the dose added to the incubation vessels. At the end of the incubation period, the incubates were sonicated, and frozen at -20°C until extracted for steroids.

Non-radioactive steroids were extracted from the medium and tissue by ether and analyzed quantitatively by radioimmunoassay after celite chromatography. The extraction, separation, and radioimmunoassay of progesterone and androstenedione (4-androstene-3,17-dione) have been previously described (1). Separation of pregnenolone and DHEA by celite chromatography was done by applying the samples in 1 ml trimethylpentane (TMP) to the column. Pregnenolone is eluted with progesterone using 4.5 ml TMP. DHEA eluted with 4 ml 5% ethylacetate in TMP (after an additional 5 ml TMP was added and the eluate discarded). Pregnenolone was measured by radioimmunoassay using Ab #11 developed by Ms. Roberta Todd at the LHRRB RIA Core facility. This antibody has a cross reaction of 14% with progesterone. The quantitative determination of both progesterone and pregnenolone in the same fraction was adjusted to account for cross reactivities of the respective antibodies. DHEA was measured by RIA using Ab #111 also developed by the RIA core facility. This antibody has 0% cross reactivity with testosterone (17ß-hydroxy-4-androsten -3-one) which elutes with DHEA off the celite column.

When ^{3}H-pregnenolone was used as a substrate, the remaining radioactive substrate and radioactive products, progesterone (P), Δ^{4}-A, DHEA, DHT (17ß-hydroxy-5 α-androstan-3-one) and testosterone (T), were separated using the following protocol. The samples were extracted with ethyl ether, dried and reconstituted with a 1:1 methylene chloride:ethanol mixture. Samples were applied to scored silica gel plates (Analab) along with standards of the 6 steroids (on channels separate from the unknowns). The plates were run 2X in a 9:1 chloroform:ether mixture. The channels with the standards were sprayed with 0.001% Direct Yellow 59 (Sigma) and the steroid zones located under UV. The silica gel:chloroform:ether system isolated progesterone Δ^{4}-A and T. Pregnenolone, DHT and DHEA co-eluted. This zone of co-eluted steroids was scraped into test tubes containing ethanol. The steroid mixture was separated from the silica gel by centrifugation and applied to aluminium oxide plates (Analab). The plates were run 2X in a 1:1 cyclohexane:ethyl acetate system which separated the three steroids. The respective steroid zones were located using standards applied to separate channels, which were sprayed with 0.001% Direct Yellow 59 and inspected under UV light. The zones containing the isolated steroid fractions were scraped off into scintillation vials containing 8 ml HP

Liquifluor (Beckman). The amount of radioactivity was converted to nanograms of steroid, since the specific activity of each dose of the substrate (^{3}H-pregnenolone) used was different.

Where applicable the data were analyzed statistically using ANOVA.

RESULTS

De Novo Production of Pregnenolone, Progesterone and DHEA by Isolated Theca and Granulosa Cells ± LH in Two Hours

Isolated granulosa cells and theca were incubated for 2 h with and without 100 ng/ml LH (Table 1). Analysis by RIA of the steroid content in media plus tissue showed that only the LH-stimulated theca produced detectable and significant levels of pregnenolone, ($P < 0.001$) and DHEA ($P < 0.001$). No progesterone was found in any of the groups, and none of the other groups produced detectable pregnenolone or DHEA.

Table 1. De novo synthesis of pregnenolone (preg), progesterone (prog), and DHEA by isolated theca and granulosa cells in vitro in 2 h incubations ± LH, n = 5.

Theca	Preg (pg)	Prog (pg)	DHEA (pg)
Control	ND[1]	ND	ND
LH (100 ng/ml)	*3390 ± 240	ND	*382 ± 46
Granulosa cells			
Control	ND	ND	ND
LH (100 ng/ml)	ND	ND	ND

Each incubate consisted of either isolated granulosa cells (50,000 cells) or theca from one preovulatory follicle. The steroids were measured by RIA and the results analyzed by ANOVA. The steroid measurement indicated by an asterisk (*) is significantly different from the respective control ($P < 0.01$).

[1] ND = non-detectable levels

Pregnenolone Conversion to Progesterone by Isolated Theca and Granulosa Cells

The conversion of exogenous non-radioactive pregnenolone

(doses of 10 to 1000 ng/ml) to progesterone was determined in 2 and 6 h incubations of isolated theca (Fig. 1A) and granulosa cells (Fig. 1B). LH was added at T_o to both the 2 and 6 h incubations. Substrate was added at T_0 to the 2 h incubations and at T_o plus 4 h to the 6 h incubations.

Thecal conversion of pregnenolone (Fig. 1A) in 2 h incubations plus/minus LH was measurable but approximately 7-10X lower than that seen by control incubations of granulosa cells (Fig. 1B). LH did not significantly stimulate the thecal conversion of pregnenolone to progesterone in 2 h incubations (Fig. 1A) but did so in granulosa cells (Fig. 1B) ($P< 0.05$).

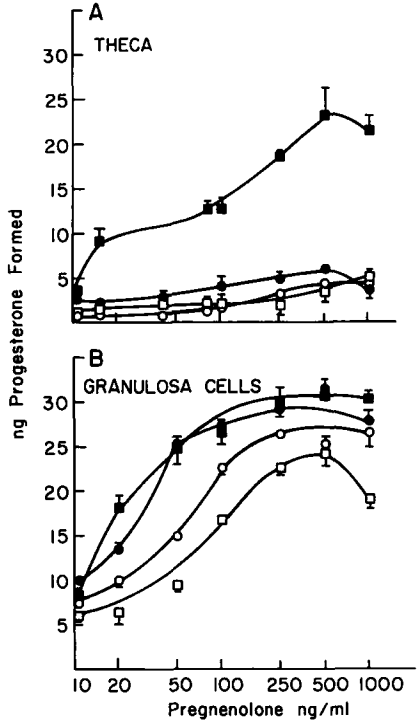

Fig. 1. Dose-response conversion of exogenous pregnenolone (10-1000 ng/ml) to progesterone in the absence or presence of LH (100 ng/ml) in 2 and 6 h incubations. Control: 2h○, 6h□. LH: 2h●, 6h■. n=4. A: theca; B: granulosa cells.

Theca that had been incubated with LH for 6 h showed significantly higher ($P < 0.01$) conversion of pregnenolone (added at T_o plus 4 h) to progesterone at all doses above 10 ng/ml (Fig. 1A).

The pregnenolone dose-response by granulosa cells in 6 h incubations was essentially the same as that seen in the respective 2 h incubations ± LH (Fig. 2B).

Conversion of DHEA by Theca in 2 and 6 H Incubations

Isolated theca were incubated for 2 and 6 h ± 100 ng/ml LH added at T_o (Fig 2). Non-radioactive DHEA was added at doses ranging from 10 to 1000 ng/ml. DHEA was added at T_o to the 2 h incubations, and at T_o plus 4 h to the 6 h incubations. The amount of Δ^4 -A found was taken as an index of degree of DHEA conversion. LH stimulated the conversion of DHEA in both 2 h and 6 h incubations ($P < 0.05$). The ability of LH to stimulate the accumulation of Δ^4-A from DHEA at 2 h is

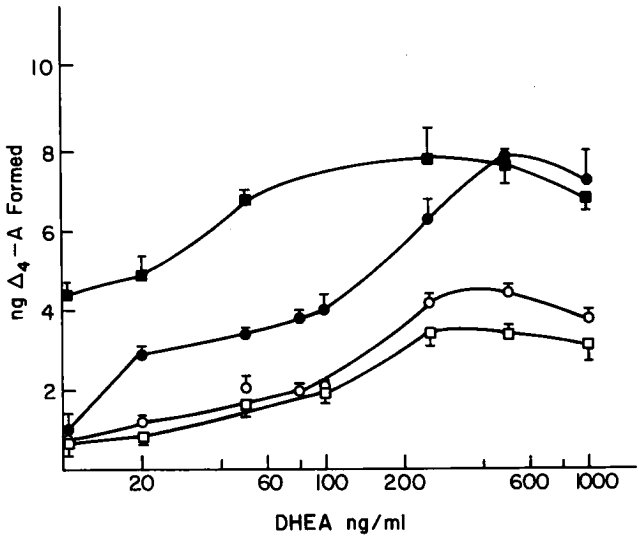

Fig. 2. Dose-response conversion of exogenous DHEA (10-1000 ng/ml) to Δ^4 -A by isolated theca in 2 and 6 h incubations ± LH (100 ng/ml). Control: 2h ○, 6h □. LH: 2h ●, 6h ■.

in contrast to the unchanged progesterone accumulation in LH-stimulated theca (Fig. 1 and Table 1).

^{3}H-Pregnenolone Dose-response in Thecal Tissue

This experiment was performed in order to determine whether the ratio of DHEA/progesterone products (a function of the relative activities of the Δ^5 and Δ^4 pathways) formed from ^{3}H-pregnenolone would change as a function of time of LH exposure. Isolated theca were divided into 4 treatment groups, 2 h control and LH-stimulated groups and 6 h control and LH-stimulated groups. Doses of 10 ng/ml (11,000 CPM/ng) , 100 ng/ml (2200 CPM/ng) and 1000 ng/ml (2200 CPM/ng) ^{3}H-pregnenolone were used. The substrate was added at T_o and T_o + 4 h to the 2 and 6 h incubates respectively.

Table 2 summarizes the data and the calculated DHEA/progesterone ratios. The amounts of progesterone, DHEA, Δ^4-A, T and DHT found were translated to total ng, because specific activity varied with dose substrate used. Control 6 h incubation data (not shown) are not essentially different from the control 2 h incubates.

It can be seen that LH at 2 h caused an increase in DHEA, Δ^4-A, T, and DHT without affecting progesterone levels. The DHEA/progesterone ratio in both 2 h control and LH-stimulated groups was over 1 (1.3 to 7.6), indicating a preferential metabolism of ^{3}H-pregnenolone through the Δ^5 rather than through the Δ^4 pathway .

The 6 h LH-stimulated groups had DHEA/progesterone ratios ranging from 0.07 to 0.24 indicating that the longer term LH effect was to significantly change the direction of ^{3}H-pregnenolone metabolism towards the Δ^4 pathway, increasing the accumulation of progesterone.

Table 2. Conversion of ^{3}H-pregnenolone to progesterone (P), androstenedione (Δ^4-A), testosterone (T), dihydrotestosterone (DHT), and DHEA by isolated theca.

Substrate (ng/ml) n = 5	P	DHEA	Δ^4-A	T	DHT	DHEA/ P
			CONTROL (2 H)			
			ng			
10	0.15	0.24	0.06	0.05	0.33	1.54
	0.11	0.15	0.17	0.06	0.13	1.33
100	0.65	1.73	1.57	0.73	3.70	2.66
	0.62	2.38	3.50	1.64	1.16	3.83
1000	1.90	11.90	5.14	14.70	17.36	6.26
	3.36	10.10	8.80	16.10	11.00	3.00
			LH (2 H)			
10	0.16	0.44	0.27	0.28	0.44	2.68
	0.22	0.38	0.34	0.15	0.57	1.72
100	0.78	3.10	12.70	2.10	lost	3.97
	1.61	4.80	6.10	3.34	lost	2.98
1000	3.82	29.00	15.80	37.10	17.80	7.59
	5.84	25.90	30.00	94.10	16.70	4.43
			LH (6 H)			
10	2.12	0.25	0.52	0.63	0.19	0.12
	2.10	0.15	0.69	0.42	0.19	0.07
100	7.50	1.23	9.39	9.49	1.23	0.16
	9.04	2.21	22.60	4.69	2.22	0.24
1000	82.30	6.77	175.59	103.87	8.21	0.08
	113.31	13.66	45.58	103.62	3.30	0.12

Incubation of the tissue was 2 or 6 h. LH was added at T_o. The substrate was added at T_o and T_o + 4 h to the 2 h and the 6 h incubations, respectively. The radioactivity found was transposed to weight of steroid product (ng). Specific activities of the ^{3}H-pregnenolone were 11,000 CPM/ng for 10 ng/ml, and 2200 CPM/ng for 100 and 1000 ng/ml. Each dose/group consisted of only 2 incubation samples. The data derived from each sample is shown.

DISCUSSION

The results of the experiments reported here support the theory that phase 1 (acute) synergistic progesterone synthesis by the hamster

follicle depends on thecal pregnenolone conversion to progesterone by granulosa cells.

Table 1 shows that theca stimulated with LH will synthesize de novo pregnenolone and DHEA with no accumulation of progesterone in 2 h incubations. Earlier studies showed an accumulation of androgens under the same experimental conditions with no detectable progesterone (1). The probability that de novo synthesized thecal pregnenolone will be preferentially metabolized through the thecal Δ^5 pathway (in the absence of granulosa cells) is indicated in the data shown in Table 2. Both non-stimulated theca and theca incubated for 2 h with LH preferentially metabolized exogenous ^{3}H-pregnenolone to DHEA as indicated by the DHEA/progesterone ratios of values greater than 1. The prolonged effect of LH was to induce significant activity of the Δ^4 pathway, which resulted in DHEA/progesterone ratios in Table 2 ranging from 0.07 to 0.24 and significant de novo synthesis of progesterone by 4 to 6 h (1).

Whether the hamster Δ^4 pathway becomes more significant due to de novo synthesis of a pregnenolone-specific 3ß-hydroxysteroid oxidoreductase or whether the existing enzyme system has an altered higher affinity for pregnenolone than DHEA is not known. There is evidence that indicates that both a pregnenolone-specific and a separate DHEA-specific 3ß-hydroxysteroid oxidoreductase can be found in bovine ovarian tissue (13). Dimino et al. found that LH can redirect a significant amount of cellular cholesterol in follicular tissue from the Δ^5 to the Δ^4 pathway (16). They found that the Δ^5 pathway was associated with existing microsomal structures and the Δ^4 pathway was associated with newly active LH-stimulated mitochondrial structures.

Evidence of LH-stimulated mitochondrial associated 3ß-hydroxysteroid oxidoreductase has been shown in ovarian tissue by several groups of investigators (17-20). YoungLai has also shown that the post-coital rabbit ovary undergoes a Δ^5 to Δ^4 shift (21).

The differential effect of LH on granulosa cell and theca (Fig. 1A and 1B) also indicates the possible differences in the respective 3ß-hydroxy steroid oxidoreductase systems. The acute effect of LH in granulosa cells was to increase pregnenolone conversion to progesterone (Fig. 1B). LH did not have the same acute effect on theca (Fig. 1A) but did stimulate thecal conversion in the 6 h incubations An acute stimulatory effect of LH on theca, however, was seen when theca were incubated with exogenous DHEA and the conversion to Δ^4 -A measured (Fig. 2).

Comparison of the relative ability of unstimulated theca (Fig. 1A) and granulosa cells (Fig. 1B) to convert pregnenolone to progesterone, shows progesterone accumulation by granulosa cells an order of magnitude higher than that seen by theca. YoungLai found the same differences in pre-coital rabbit granulosa cells and theca (21). In our studies, the relative differences between theca and granulosa cells were maintained after LH stimulation during 2 h incubations but were not evident in the 6 h incubations where thecal conversion of pregnenolone to progesterone was increased significantly ($P< 0.01$) (Fig. 1A and 1B).

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institutes of

Child Health and Human Development, RO1 HD 14709-01A1. We would like to thank the LHRRB RIA core facility, under the direction of Ms. Roberta Todd for providing RIA materials and facilities.

REFERENCES

1. Makris, A. and Ryan, K.J., Endocr. Res. Comm. 4(3+4), 233 (1977).
2. Makris, A. and Ryan, K.J., Abstr. # 29, 6th International Congr. on Hormonal Steroids, Jerusalem, Israel, 5-10 Sept., 1982.
3. Makris, A. and Ryan, K.J., (1983), submitted to Endocr. Res. Comm..
4. Turgeon, J.and Greenwald, G.S., Endocrinology 90, 657 (1972).
5. Gougeon, A., Ann. Biol. Anim. Biochem. Biophys. 17(6), 1087 (1977).
6. Pupkin, M., Bratt, H., Weisz, J., Lloyd, C.W. and Balogh, K., J. Endocrinology 79, 316 (1976).
7. Bjersing, L., Histochemie 10, 295 (1967).
8. Ikonen, M., Niemi, H. Pesonen, S., and Timonen, S., Acta Endocr. (Kbh) 38, 293 (1961).
9. Rubin, B.L., Hamilton, J.A. and Driks, E.C., Endocrinology 72, 924 (1963).
10. Blaha, G.C. and Leavitt, W.W., Biol. Reprod. 3, 362 (1970).
11. Ryan, K.J. and Smith, O.T., Rec. Prog. Hormone Res. 21, 285 (1965).
12. Somma, M., Sander, T. and Lauthier, A., J. Clin. Endocrinol. Metab. 29, 457 (1969).
13. Gibb, W. and Hagerman, D.D., Steroids 28, 31 (1976).
14. Mahajan, D..K. and Samuels, L.T., Steroids 24, 713 (1974).
15. Ward, M.C., Anat. Rec. 94, 139 (1946).
16. Dimino, M.J., Elfont, E.A. and Berman, S.K., Adv. Exp. Med. Biol. 112, 505 (1978).
17. Westwerdt, W., Muller, O. and Brandau, H., Adv. Exp. Med. Biol. 112, 123 (1978).
18. Crisp, T.M. and Channing, C.P., Biol. Reprod. 17, 55 (1972).
19. Peluso, J.J., Steger, R.W. and Hatz, E.S.E., Biol. Reprod. 16, 600 (1977).
20. Shuchner, E.B. and Stockert, J.C., Protoplasma 76, 133 (1973).
21. YoungLai, E.V., Acta Endocrinol. (Kbh) 74, 775 (1973).