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Inhibition of Leydig Cell Steroidogenesis: Effect of Actinomycin D Before and After Preincubation of Leydig Cells *In Vitro*

By

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The effect of preincubating purified Leydig cells in Eagle's medium and the subsequent effect of the mRNA synthesis inhibitor actinomycin D on LH-stimulated testosterone synthesis has been investigated. The inhibitory effect obtained was found to decrease with the period of preincubation; with 0, 1, 2 and 3 h preincubation before the addition of LH (100 ng/ml) 36.5 ± 3.9, 31.2 ± 2.5, 17.8 ± 3.8 and 13.6 ± 2.9 % inhibition occurred respectively when actinomycin D (6.4 μM) was added and the cells were incubated for 2 h (means ± SEM, n = 5). During the first hour of incubation with LH and actinomycin D no inhibition occurred in cells that had been preincubated for 3 h.

These results suggest that during preincubation and independently of LH, synthesis of intermediates (possibly mRNA(s)) required for stimulation of steroidogenesis may take place and that subsequent stimulation of steroidogenesis by LH occurs without further *de novo* mRNA synthesis.

Key words: Leydig cells – actinomycin D – steroidogenesis – LH – preincubation – inhibition.

It has previously been shown that inhibitors of RNA and protein synthesis can inhibit luteotropin (LH)-stimulated testosterone production in rat testis Leydig cells *in vitro*, suggesting that the LH stimulation of the synthesis of mRNA and proteins may be involved in the action of LH on steroidogenesis in the testis (Cooke et al. 1975a,b; Mendelson et al. 1975).

Further work showed that if Leydig cells isolated from rat testes are preincubated for 2 h in the absence of LH, then the properties of these cells with respect to their response to LH changes. For example, the time required to detect LH-stimulated testosterone synthesis is less than 5 min in preincubated cells compared with 30 min for the non-preincubated cells (Cooke et al. 1977). As it is possible that these changes during preincubation may involve new mRNA and protein synthesis which occur independently of LH, the effect of actinomycin D, a mRNA synthesis inhibitor, on the LH-stimulated testosterone synthesis in preincubated and non-preincubated Leydig cells has now been investigated. This has been carried out by preincubating purified rat testes Leydig cells for different times (0–5 h) followed by incubation with LH in the presence and absence of actinomycin D and measurement of the testosterone production.

Materials and Methods

Leydig cell suspensions from rat testes were prepared and purified by centrifugation through Ficoll and Dextran solutions as described previously (Janszen et al. 1976). The cell suspensions ($0.2–0.4 \times 10^6$ cells, containing approximately 60 % Leydig cells) were preincubated in Eagle's medium (0.1 ml) containing 0.1 % bovine serum albumin for different periods of time at 32°C with continuous shaking under an atmosphere of $O_2 + CO_2$ (95:5), as indicated in the text. LH (final concentration 100 ng/ml) and actinomycin D (final concentration 6.4 μM) were then added in 10 μl concentrated solutions as indicated and the incubations were continued for different periods of time. After incubation testosterone was extracted and determined as previously described (Verjans et al. 1973).

Incorporation of [^{3}H]uridine into Leydig cell RNA was measured as described by Grootegoed et al. (1977) after incubation of the preincubated and non-preincubated cells for 2 h with [5-^{3}H]uridine (27.6 μCi/mM, 0.5 μCi/incubation).

For statistical analysis the Student's t-test for correlated data was used.

Results

Leydig cells were preincubated for different times (0–5 h) followed by incubation for 2 h with LH (100 ng/ml) in the presence and absence of actinomycin D (6.4 μM). The testosterone formed during the latter incubations is given in Fig. 1. It was found that the inhibitory effect of actinomycin D was dependent on the time of preincubation; in the non-preincubated cells the LH-stimulated testosterone production was decreased by 38 % in the presence of actinomycin D compared with only 5–7 % after 3–5 h preincubation.

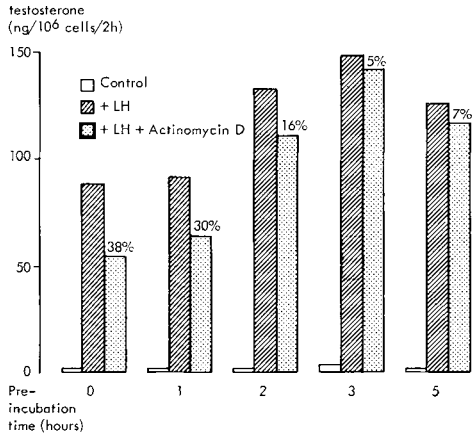

Fig. 1.

Effect of preincubation time on the inhibition of LH-stimulated testosterone synthesis by Actinomycin D in testis Leydig cells. The Leydig cells were preincubated for 0–5 h at 32°C as indicated. Incubations were then continued after the addition of LH (100 ng/ml) in the absence and presence of actinomycin D (6.4 μM) or without any additions (controls). After 2 h incubation testosterone was extracted and measured by radioimmunoassay. The percentages of inhibition of LH-stimulated testosterone synthesis by actinomycin D are indicated. The results are means of duplicate incubations.

In a series of 5 experiments with cells preincubated for 0, 1, 2 and 3 h before addition of LH (100 ng/ml) 36.5 ± 3.9, 31.2 ± 2.5, 17.8 ± 3.8 and 13.6 ± 2.9 % respectively inhibition of LH-stimulated testosterone synthesis occurred when actinomycin D (6.4 μM) was added with the LH (means ± SEM). The values obtained for 1, 2 and 3 h preincubation were all significantly different from the non-preincubated cells ($P < 0.05$, 0.005 and 0.005 respectively). Under the same conditions 80 % inhibition of the incorporation of [^{3}H]uridine into RNA occurred in the presence of actinomycin D in the preincubated (3 h) as well as in the non-preincubated cells.

In cells that had been preincubated for 3 h the effect of time on the inhibition of LH-stimulated testosterone production by actinomycin D was measured (Fig. 2). After 1 h incubation no inhibition occurred. After 2 or more h incubation the degree of inhibition increased reaching 34 % after 5 h incubation.

Discussion

In a previous study carried out with rat testis Leydig cell preparations it was reported that addition of the RNA synthesis inhibitors actinomycin D (10 μM) and cordycepin (1 mM) completely inhibited hCG-stimulated testosterone syn-

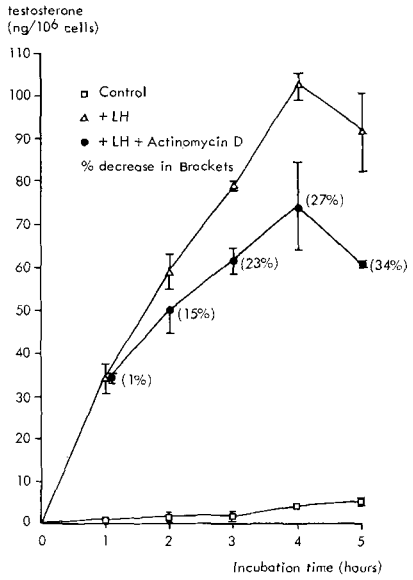

Fig. 2.

Effect of time on LH-stimulated testosterone synthesis in preincubated Leydig cells in the presence and absence of Actinomycin D. The Leydig cells were preincubated for 3 h at 32°C. Incubations were then continued after additions as indicated in the text to Fig. 1. After different times of incubation, testosterone was extracted and measured by radioimmunoassay. The percentages of inhibition of LH-stimulated testosterone synthesis by actinomycin D are indicated. The results are means and ranges of duplicate incubations.

thesis (Mendelson et al. 1975). In similar experiments with rat testis interstitial tissue and actinomycin D (6 μM) under conditions in which 90 % of the RNA synthesis was inhibited, approximately 50 % inhibition of LH-stimulated testosterone production was obtained (Cooke et al. 1975a). It is apparent from the present study that the degree of inhibition by actinomycin D of LH-stimulated steroidogenesis can be reduced to a very low level if the cells are preincubated before addition of LH. Furthermore, even this low level of inhibition does not become apparent until after 1 h of incubation of the preincubated cells with LH and actinomycin D. These results suggest that during the preincubation period carried out in the absence of added LH, synthesis of intermediates, possibly mRNA(s) required for protein(s) involved in steroidogenesis, may be taking place. Initial stimulation of steroidogenesis in the preincubated cell with LH then proceeds independently of mRNA synthesis. Evidence obtained previously on the effect of preincubation on the kinetics of LH stimulation of testosterone synthesis (Cooke et al. 1977) also agrees with this hypothesis; in preincubated cells LH stimulates testosterone synthesis much more rapidly

(within 5 min) than in non-preincubated cells (30 min required). In addition the time taken to detect stimulation of testosterone production in the preincubated cells, in contrast to the non-preincubated cells, is too short to allow *de novo* mRNA and protein synthesis.

Further work is required to determine the nature of the events which are occurring during the preincubation period. However, it is possible that the production of the proposed mRNA(s) and proteins(s) is simply part of a general repair process of Leydig cells which may have been partly damaged during their preparation and purification. Preliminary evidence indicates that this is not primarily due to changes in the LH receptors because the effects of preincubation on actinomycin D inhibition were also found when the cells were stimulated with dibutyryl cyclic AMP in place of LH.

To conclude, the results obtained in this study suggest that during the preincubation period synthesis of intermediates, possibly mRNA(s), occurs which are required for steroidogenesis. This process is apparently independent of LH. In the preincubated cells LH initially stimulates testosterone synthesis without prior *de novo* mRNA synthesis.

References

Cooke B. A., F. H. A. Janszen & H. J. van der Molen (1975a) Effect of RNA and protein synthesis inhibitors on testosterone biosynthesis in testis Leydig cell preparations. *Acta endocr. (Kbh.) Suppl. 199, 104.* Abstract No. 41.

Cooke B. A., F. H. A. Janszen, W. F. Clotscher & H. J. van der Molen (1975b) Effect of protein synthesis inhibitors on testosterone production in rat testis interstitial tissue and Leydig cell preparations. *Biochem. J. 150,* 413.

Cooke B. A., L. M. Lindh & F. H. A. Janszen (1977) Role of cyclic AMP in steroidogenesis in Leydig cells. Discrepancies between effects of luteinizing hormone and cholera toxin. *Febs letters 73,* 67.

Grootegoed J. A., A. H. Grollé-Hey, F. F. G. Rommerts & H. J. van der Molen (1977) Ribonucleic acid synthesis *in vitro* in primary spermatocytes isolated from rat testis. *Biochem. J. 168,* 23.

Janszen F. H. A., B. A. Cooke, M. J. A. van Driel & H. J. van der Molen (1976) Purification and characterization of Leydig cells from rat testis. *J. Endocr. 70,* 345.

Mendelson C., M. Dufau & K. Catt (1975) Dependence of gonadotropin-induced steroidogenesis upon RNA and protein synthesis in the interstitial cells of the rat testis. *Biochim. biophys. Acta 411,* 222.

Verjans H. L., B. A. Cooke, F. H. de Jong, C. M. M. de Jong & H. J. van der Molen (1973) Evaluation of a radioimmunoassay for testosterone estimation. *J. Steroid Biochem. 4,* 665.

DISCUSSION

Drosdowsky: Question directed to Dr. Cooke. Do you have an idea on the nature of the protein molecules you have isolated by slab gel electrophoresis in immature rats?

Cooke: No, not in the immature rat. In the mature rat testis Leydig cell the LH induced protein with a mol. wt. of 21 000 is specific for the Leydig cell and is located in the soluble fraction of the cell. Its synthesis is not stimulated by testosterone or FSH. The phosphoproteins whose phosphorylation is stimulated by LH have been partially characterized. The 14 000 mol. wt. protein is associated with the nucleus and the 58 000 mol. wt. protein is soluble and may be the type II R subunit of the cyclic AMP dependent protein kinase.

Dufau: It was of interest to learn that you could reduce the lag period of testosterone response to LH from 30 to 5 min or less by preincubating the Leydig cells before stimulation with trophic hormone. Since we have recently demonstrated that there is no lag period for pregnenolone stimulation by hCG (Cigonaga S., M. L. Dufau & K. J. Catt (1978) J. biol. Chem. June issue), while the testosterone lag period was 20 to 30 min in these experiments. Combining your results and our results we could conclude that the preincubation of Leydig cells would affect steps following pregnenolone production.

Means: Have you utilized 2-dimensional gel electrophoresis to examine the total number of specific proteins stimulated by LH in your system?

Cooke: We are in the process of doing this. The proteins we have isolated so far which give a single band on one dimensional gel electrophoresis may well be a mixture of proteins.

Means: Only 2 % of total RNA synthesized in a cell is mRNA. Actinomycin D preferentially affects rRNA synthesis. In your experiments this drug inhibited uridine incorporation by 90 %. Therefore, have you any direct evidence that mRNA synthesis is or is not involved in the acute effects of LH?

Cooke: No, our experiments only indicate that an actinomycin D sensitive step is influenced by preincubation; after 3 h preincubation no effect of actinomycin D can be detected during the first hour of incubation with LH. Cordycepin inhibition of LH-stimulated steroidogenesis was similarly affected by preincubation.

Santen: In collaboration with Drs. Cohn, Misbin, Samojlik and Foltz, we have administered aminoglutethimide (AG) acutely to normal adult men in order to study its effects on testicular steroidogenesis. Sixteen subjects between the ages of 21 and 30 received either placebo or 1250 mg of AG in divided doses during a 24 h period. In order to examine testicular steroids predominantly, adrenal function was inhibited by the administration of dexamethasone (2 mg) to all subjects on the night of the experiment. AG abolished the normal diurnal rise in testosterone and significantly suppressed the levels of this steroid at 07.00 and 09.00 h. Plasma estrone and estradiol concentrations were similarly lowered in men given AG. As a result, LH and FSH levels were higher in men receiving AG, although the data exhibited a large variance due to pulsatile gonadotropin secretion. Further study is required to define fully the long term effects of this drug on the pituitary-gonadal axis and on testosterone sensitive aspects of spermatogenesis.

Sherins: How long was aminoglutethimide administered to the normal men? The increase in LH and FSH following the decrease in aromatization of T to E_2 and E_1 without a reciprocal increase in T might be due to the very acute exposure of these men to the aromatase inhibitor.

Santen: Aminoglutethimide was administered over 12 h. Consequently the secondary effects of increasing LH and FSH levels on testosterone secretion may not have been observed due to the acute nature of the study. Longer term studies are necessary to evaluate this issue.

Payne: Aminoglutethimide besides being an inhibitor of cholesterol side chain cleavage also is a potent inhibitor of aromatase.

Santen: We are aware of the effects of aminoglutethimide on aromatase activity. We have examined the effects of this drug on aromatization in human females and shown a 95 % inhibition of this reaction using isotopic kinetic techniques. The slightly greater reduction of estradiol than testosterone observed in this study may reflect this action.