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Effects of Oestrogens and FSH on LH Stimulation of Steroid Production by Testis Leydig Cells from Immature Rats

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Hypophysectomy of immature male rats results after 5 days in a decreased production of testosterone by isolated testis Leydig cells in response to LH. The LH responsiveness of the Leydig cells can be partly restored by treatment of the hypophysectomized rats with FSH. In continuation of previous reports on this subject (*Steroids* 28 (1976) 847; and 30 (1978)) the following conclusions were derived from the results in the present paper:

1. After hypophysectomy of immature male rats the production of testosterone (T) as well as of 5-pregnenolone (Δ 5P) by isolated Leydig cells in response to LH is reduced.
2. Daily administration of FSH after hypophysectomy restores the Δ 5P production in response to LH almost completely, but has a much smaller effect on the restoration of T production.
3. Administration of oestradiol benzoate (E_2B) together with FSH has no effect on the restoration of LH-stimulated Δ 5P production, but causes a reduction of T production, when compared with Leydig cells from animals treated with FSH only.
4. Treatment of intact immature rats with E_2B results in a decreased production of T and an increased production of Δ 5P in isolated Leydig cells.
5. From experiments with labelled pregnenolone it appears that E_2B and diethylstilboestrol (DES) inhibit the 17 α -hydroxylase activity of Leydig cells from intact as well as from hypophysectomized rats. This results

in a reduced conversion of pregnenolone to C_{19} -steroids and in increased production of 3α -hydroxy- 5α -pregnan-20-one from $\Delta 5P$.

6. The observed effects of FSH and E_2 were similar within a dose range of 100–10 000 ng LH per 10^6 Leydig cells.

Key words: steroid 17α -hydroxylase testis – steroid production – oestrogen – FSH and LH effects.

The stimulation of testosterone production by LH in isolated Leydig cells from immature rats appears to be dependent on the previous presence of other trophic hormones. Hypophysectomy results within 5 days in a loss of the ability of isolated Leydig cells to produce testosterone in response to LH, whereas daily treatment with FSH partly restores the testosterone producing capacity of the isolated cells. Also oestradiol might play a role in the regulation of testosterone production since simultaneous administration of oestradiol with FSH appears to abolish the effect of FSH on the testosterone producing capacity of Leydig cells under the influence of LH (van Beurden et al. 1976).

Testosterone production is the result of testosterone synthesis and conversion. The use of testosterone production as a parameter for the LH stimulation of the steroidogenic activity of Leydig cells from immature rats could give a wrong impression due to the high conversion of testosterone to 5α -reduced metabolites. Therefore, a better parameter of the steroidogenic capacity of Leydig cells from immature rats could be the estimation of pregnenolone, which is the obligatory intermediate in the formation of androgens, or the estimation of "total androgens" as the sum of testosterone and testosterone metabolites.

In the present study we have tried to elucidate which step in testicular steroidogenesis is affected by hypophysectomy, FSH or FSH plus oestradiol treatment of immature rats. We have estimated in isolated Leydig cells both pregnenolone production and androgen production with antisera raised against testosterone or against total 17β -hydroxy- C_{19} steroids.

Materials and Methods

Animals

All rats used were from the Wistar substrain R-Amsterdam. Leydig cells were isolated from the testes of 28–31 days old rats. Hypophysectomy of the rats was performed on day 23–25. Administration sc of FSH (60 μ g/day) and/or oestradiol-benzoate (E_2B) (5 μ g/day) was started on the day after hypophysectomy and was continued for 5 days. Intact control animals were from the same age as the hypophysectomized rats.

[1,2,6,7-³H]Testosterone, spec. act. 100 Ci/mmol, was purchased from the Radiochemical Centre, Amersham, England. [16-³H]Pregnenolone was obtained from CEN, Mol, Belgium. Unlabelled steroids were purchased from Steraloids, Pawling, USA and were used without further purification. Cyanoketone (2 α -cyano-4,4'-17 α -trimethyl-17 β -hydroxy-5-androsten-3-one), an inhibitor of 3 β -hydroxysteroid dehydrogenase activity, is a product of Stirling-Winthrop, New York, USA. SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl)-1(2H)-naphthalenone), an inhibitor of 17 α -hydroxylase activity, was a gift from Ciba-Geigy, Basel, Switzerland.

Testosterone was measured according to the method described by Verjans et al. (1973). Pregnenolone was determined as described by Van der Vusse et al. (1975). "Total androgens" were measured with an antiserum raised against 17 β -OH-androstane steroids. The antiserum was a gift from Dr. K. Purvis, Oslo. A 1:20 dilution of the antiserum was stored at -20°C. Just before use the antibody was diluted to 1:25 000 with 0.01 M phosphate buffer pH 7.4, containing 0.2 % gelatine. After incubation with extracted steroids for 16 h at 4°C, bound and unbound steroids were separated with dextran coated charcoal. [³H]Testosterone was used as indicator ligand and it was found, that 100-500 pg added testosterone, dihydrotestosterone and 5 α -androstane-3 α /3 β ,17 β -diols would compete for binding of the [³H]testosterone. No displacement of labelled testosterone was observed with 200-400 pg androsterone or 5 α -androstane-3,17-dione. Results are expressed relative to the amount of testosterone required for a comparable displacement of [³H]testosterone.

Materials and methods not described in the present paper are the same as described previously by Van Beurden et al. (1976, 1978).

Results

Estimation of pregnenolone production

If pregnenolone is used as a parameter for Leydig cell steroid production, it is necessary to inhibit the conversion of pregnenolone to other steroids, because pregnenolone can be converted to progesterone or 17 α -hydroxypregnenolone by 3 β -hydroxysteroid dehydrogenase and 17 α -hydroxylase, respectively.

Incubation of Leydig cells for 2 h with labelled pregnenolone in the presence of 5 μ M cyanoketone and analysis of the labelled steroids using paper chromatography (Bush AII) showed that all the labelled steroid found could be identified as 17 α -hydroxypregnenolone. Under these conditions the 3 β -hydroxysteroid dehydrogenase activity was completely inhibited without affecting pregnenolone production.

In order to inhibit 17 α -hydroxylase activity Leydig cells were incubated

with 5 μM cyanoketone and increasing amounts of SU-10603. After 2 h incubation with or without LH both conversion of labelled pregnenolone and production of pregnenolone were measured. At a concentration of 19 μM SU the amount of pregnenolone produced was maximal and the recovery of [^3H]pregnenolone was 60 %. At higher concentrations of SU pregnenolone recovery was higher, but pregnenolone synthesis was inhibited at these concentrations. Pregnenolone synthesis in Leydig cells was measured routinely, therefore, after incubation of cells in the presence of 5 μM cyanoketone and 19 μM SU.

The effect of hypophysectomy, FSH and oestradiol benzoate treatment on pregnenolone, testosterone and "total androgen" production

Rats were hypophysectomized at day 23. During 5 days rats were injected with FSH, FSH plus E_2B or vehicle only. At day 29 the hypophysectomized rats and intact rats of the same age were killed and Leydig cells were prepared. In order to evaluate pregnenolone production, part of the Leydig cells were incubated in the presence of cyanoketone and SU with or without 200 ng LH/ml. Another part of the cell preparation was incubated without inhibitors for the determination of testosterone and "total androgens". Results are presented in Table 1.

Table 1.

Steroid production in Leydig cells isolated from intact, hypophysectomized (hypox), hypophysectomized FSH treated and hypophysectomized FSH plus oestradiol benzoate (E_2B) treated immature rats. Results given as mean \pm sd for 7-14 independent experiments.

Steroid	LH (ng/ml/ 10^6 cells)	Steroid production (ng/ 10^6 cells/2 h)			
		Intact rats	Hypox rats	Hypox + FSH rats	Hypox + FSH + E_2B rats
Pregnenolone	0	0.73 \pm 0.73	0.09 \pm 0.14	0.44 \pm 0.49	0.59 \pm 0.31
	200	23.26 \pm 11.17	6.43 \pm 4.43	18.32 \pm 5.37	21.98 \pm 10.33
Testosterone	0	0.15 \pm 0.17	0.05 \pm 0.10	0.16 \pm 0.19	0.25 \pm 0.27
	200	4.29 \pm 1.62	0.62 \pm 0.38	1.40 \pm 0.50	0.70 \pm 0.55
Total androgens	0	0.30 \pm 0.31	0.31 \pm 0.50	0.17 \pm 0.21	0.20 \pm 0.26
	200	14.57 \pm 7.04	1.70 \pm 0.88	3.25 \pm 1.55	2.00 \pm 1.78

From these results it can be concluded that:

1. hypophysectomy resulted in a lower capacity of Leydig cells to produce pregnenolone and androgens;
2. FSH treatment of hypophysectomized rats resulted in a restoration of pregnenolone synthesizing capacity, however, androgen production was still partly inhibited.
3. oestrogen treatment in addition to FSH had no inhibitory effect on pregnenolone production. However, testosterone and total androgen production in FSH + E₂B treated hypophysectomized rats were significantly lower ($P < 0.05$) than in hypophysectomized rats treated with FSH only. Because the oestradiol treatment does not influence testosterone metabolism, E₂B seems to have an inhibitory effect on one or more of the enzymes involved in the conversion of pregnenolone to testosterone.

Effect of oestradiol benzoate on testicular steroid production in intact immature rats

In order to test whether the effect of E₂B was restricted to hypophysectomized rats, we have also investigated the effect of E₂B on intact immature rats. From the results in Table 2 it appears, that after addition of different amounts of LH to Leydig cells from E₂B-treated rats testosterone production was always smaller than in comparable cells from untreated rats, whereas the pregnenolone production in cells from E₂B rats was always higher than in cells from control rats. These results again support an effect of E₂B on some step in the conversion of pregnenolone to testosterone, resulting in decreased testosterone production and increased pregnenolone production.

Table 2.

Effect of different amounts of LH (0–10 000 ng/ml) on steroid production (ng/10⁶ cells/2 h) in Leydig cells isolated from testes of immature rats and from immature rats treated with E₂B (5 µg/day for 5 days). Results are mean values of duplicate experiments.

Treatment	ng LH/ml/10 ⁶ cells				
	0	10	100	1000	10 000
<i>Pregnenolone production</i>					
Control	1.39	33.8	23.3	22.2	18.8
E ₂ B	2.67	95.1	81.6	81.2	70.0
<i>Testosterone production</i>					
Control	0.09	3.94	3.96	5.01	5.10
E ₂ B	0.26	2.22	2.31	2.57	2.28

Effect of different amounts of LH on steroid production in Leydig cells from testes of immature intact and hypophysectomized rats

The oestrogen treatment might have caused a change in the total steroid producing capacity of the Leydig cells, or in the sensitivity of the Leydig cells to LH. Therefore we have investigated the effect of different amounts of LH on the Leydig cells. From the results in Table 2 and 3 it appears, that the effect of E₂B is quantitatively comparable with all dosages of LH used. It is unlikely, therefore, that the estrogen treatment would have influenced the sensitivity of the Leydig cells for LH within the range of 10–10 000 ng/10⁶ cells.

Metabolism of pregnenolone by Leydig cells isolated from testes of intact and hypophysectomized immature rats

In order to investigate which step(s) in the conversion of pregnenolone to androgens was affected by the different hormonal treatments, isolated Leydig cells from either intact, hypophysectomized, and FSH or FSH plus E₂B treated

Table 3.

Effect of different amounts of LH (0–10 000 ng/ml) on steroid production (ng/10⁶ cells/2 h) in Leydig cells isolated from testes of immature rats, hypophysectomized immature rats (hypox) and hypophysectomized immature rats treated with FSH or FSH plus E₂B. Results are the mean values of duplicate experiments.

Treatment	ng LH/ml/10 ⁶ cells				
	0	10	100	1000	10 000
<i>Pregnenolone production</i>					
Intact	2.3	43.1	29.5	24.7	31.4
Hypox	2.2	–	10.4	10.4	7.2
Hypox + FSH	1.0	48.0	30.8	35.7	29.2
Hypox + FSH + E ₂ B	1.5	29.8	26.8	18.6	18.0
<i>Testosterone production</i>					
Intact	0.08	2.37	3.05	3.40	3.14
Hypox	0.18	–	0.75	0.79	0.76
Hypox + FSH	0.11	1.39	1.28	2.20	1.67
Hypox + FSH + E ₂ B	0.17	0.84	0.75	0.89	1.08
<i>Total 17β-hydroxy androgens</i>					
Intact	0	8.83	8.92	10.5	9.25
Hypox	0	–	2.28	2.20	2.40
Hypox + FSH	0	4.62	4.25	5.24	5.19
Hypox + FSH + E ₂ B	0	2.13	2.11	2.49	3.08

rats were incubated with [^3H]pregnenolone. At time 0', 30' and 60' the reaction was stopped by addition of ethylacetate and the extracted steroids were submitted to thin layer chromatography on silica gel (solvent system dichloromethane:ether 85:15) and paper chromatography (Bush AII). The chromatographic pattern of radioactive metabolites was similar for all incubations, with the exception of the cells from E_2B -treated animals. During the latter incubations an extra metabolite was formed, which was subsequently identified as 3α -hydroxy- 5α -pregnane-20-one. The same metabolite was also found after incubation with pregnenolone of Leydig cells from intact immature rats treated with E_2B or diethylstilboestrol. We have never observed this metabolite in cells from rats not treated with oestrogens.

From the results of steroid metabolism it was concluded, that:

1. pregnenolone is converted in cells from intact rats to androstanediol and androsterone (ratio androsterone:androstanediol = 1:1);
2. in untreated or FSH-treated hypophysectomized rats pregnenolone is mainly converted to androsterone (ratio androsterone:androstanediol = 5:1);
3. after E_2B treatment of intact as well as hypophysectomized FSH-treated rats 3α -hydroxy- 5α -pregnane-20-one could be identified as a metabolite of pregnenolone;
4. because treatment of intact or hypophysectomized immature rats with E_2B or diethylstilboestrol results in the appearance of 3α -hydroxy- 5α -pregnane-3-one, it is likely that the estrogen treatment has an inhibitory effect on 17α -hydroxylation of pregnenolone

Discussion

In a previous study (Van Beurden et al. 1976) we used only testosterone production as a parameter for testicular steroidogenesis. The observed changes in testosterone production after different hormonal treatments might be the result of changes in testosterone synthesis as well as conversion. Therefore measurement of the formation of total androgens including 5α -reduced metabolites of testosterone or the determination of pregnenolone, the precursor of androgens, were considered to be better parameters for the steroidogenic capacity of isolated Leydig cells. The steroidogenic activity (see Table 1) in the different cell preparations appeared always highest when pregnenolone was used as a parameter. The use of the antibody raised against 17β -OH- C_{19} steroids gives a higher estimation of the steroidogenic activity than the estimation of testosterone. However, when [^3H]testosterone or [^3H]pregnenolone were incubated with isolated Leydig cells, the main metabolites were androsterone and andro-

stanediol (data not shown). Since the antibody raised against 17β -OH- C_{19} steroids does not bind androsterone still part of the formed androgens will not be measured using this antibody.

When testosterone, pregnenolone and "total androgens" were measured after incubation of isolated Leydig cells from intact, untreated hypophysectomized and FSH or FSH plus E_2B treated hypophysectomized rats, it was observed (Table 1) that hypophysectomy results in a decreased pregnenolone synthesis. This could be due to a loss of LH receptors (Chen et al. 1977) or to a loss in cytochrome P-450 which is necessary for the activity of cholesterol side-chain cleaving enzyme (Purvis et al. 1973). After administration of FSH to hypophysectomized rats the pregnenolone production was at the level of the production in cells from intact rats. Testosterone and "total androgen" production, however, were still significantly lower than in cells from intact rats, which could result from a decrease in the activity of several enzymes, such as 17α -hydroxylase, $17-20$ lyase or 17β -hydroxysteroid dehydrogenase (Purvis et al. 1973; Shikita & Hall 1976) after hypophysectomy.

The inhibitory effect of E_2B on LH-stimulated testosterone production appears to be the result of inhibition of one or more of the enzymes involved in the conversion of pregnenolone to testosterone, since no difference in pregnenolone synthesis of FSH-treated and FSH plus E_2B treated hypophysectomized rats could be observed. Total androgens and testosterone production, however, were always inhibited by E_2B . Similar results were obtained with Leydig cells isolated from intact rats and from Table 3 it appears that E_2B treatment of intact rats results in reduced testosterone production and in increased pregnenolone production. For the intact rats it cannot be excluded that E_2B treatment might have influenced LH/FSH levels, which might be (partly) responsible for the high steroid production.

In order to investigate the specific step(s) in the steroidogenic pathway affected by the different hormonal treatments, the conversion of labelled pregnenolone was investigated. It was found that the main metabolites were androsterone and androstanediol. In cells from hypophysectomized rats less androstanediol was formed and androsterone was the main metabolite. Treatment of hypophysectomized rats with FSH plus E_2B or of intact immature rats with E_2B or DES resulted in the formation of 3α -hydroxy- 5α -pregnan-20-one as a metabolite of labelled pregnenolone. Under these conditions hardly any 17α -hydroxylated metabolites of pregnenolone or progesterone or C_{19} steroids were formed. Hence, it was concluded that oestrogen treatment resulted in a reduction of 17α -hydroxylase activity for conversion of pregnenolone. Our observations that pregnenolone can be metabolized via 5α -pregnane-steroids are in agreement with results from Tsujimara & Matsumoto (1974), Moger & Armstrong (1974) and Mitzutani et al. (1977), who showed that progesterone metabolism by testes from prepubertal rats, in contrast to adult rats, resulted in a

large conversion to 5α -pregnane- $3\alpha,17\alpha$ -diol-20-one. A reduction of testicular 17α -hydroxylase activity after oestrogen treatment *in vivo* has also been shown in hCG-treated hypophysectomized male rats (Kremers et al. 1977) and in long-term oestrogen-treated human males (Rodriguez-Rigau 1977). *In vitro* addition of large amounts (5–50 $\mu\text{g/ml}$) of oestrogens during incubation of decapsulated testis tissue from mice also appears to cause a decreased testosterone production (Bartke et al. 1977); the physiological significance of such high amounts of oestrogens is not clear, however.

There are indications that oestradiol can inhibit also other testicular enzyme activities involved in steroid biosynthesis. Kalla et al. (1977) have reported that subcutaneous implantation of oestrogen in hCG- and hFSH-treated hypophysectomized rats caused a decrease in testicular testosterone content, and an increase in testicular progesterone as well as 17α -hydroxyprogesterone. It was concluded from these results that oestradiol had reduced the 17–20 desmolase activity required for conversion of 17α -hydroxyprogesterone to androstenedione. These results were obtained with adult rats and it remains to be investigated if oestrogen treatment of immature and mature male rats results in a reduction of different enzyme activities, i. e. 17α -hydroxylase or 17,20-lyase, in testis Leydig cells.

Summarizing our results it can be concluded that the use of testosterone as a parameter for steroidogenesis in Leydig cells from immature rats gives an underestimation of the steroidogenic activity of the cells. A better parameter is the determination of pregnenolone. Hypophysectomy of immature rats results in a decrease of pregnenolone synthesis, which can be restored by administration of FSH, probably via some factor required for pregnenolone production. Oestrogen treatment of intact as well as hypophysectomized FSH-treated immature rats results in an inhibition of 17α -hydroxylase activity, causing decreased testosterone production and increased pregnenolone production in isolated Leydig cells.

Acknowledgment

The authors are grateful to Dr. K. Purvis, Oslo, for the gift of antiserum against 17β -hydroxy-androstane steroids.

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DISCUSSION

Troen: There are other enzymatic steps that may be affected by estrogens. We have reported (*J. Clin. Endocr. and Metab.* 34, 968, 1972) using human testicular tissue that estradiol added *in vitro* has an inhibitory effect on 3 β -hydroxysteroid dehydrogenase.

Van der Molen: We have not ourselves investigated human tissue, but as we discussed in our manuscript, from Dr. Steinberger's group (Rodriguez et al. 1977) there is a report that long term administration of estrogens to human males causes a reduction of 16 α -hydroxylase activity. For male rats it has been reported also that C17-20 lyase activity could be inhibited after estrogen administration.