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STIMULATION OF THE SYNTHESIS OF STEROIDS AND STEROID SULPHATES IN HUMAN TESTICULAR TISSUE *IN VITRO* BY hCG AND BY 8-BROMO-CYCLIC AMP

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Summary—Small pieces (10–20 mg) of human testis tissue were incubated for 4 h in the presence or absence of hCG and 8-bromo-cAMP and the concentrations of testosterone, some of its steroidal precursors, and their sulphates were measured by radioimmunoassays. The results showed, we believe for the first time, that the production of steroid sulphates as well as of unconjugated steroids can be stimulated in human testis tissue *in vitro* and they confirm earlier observations *in vivo* which suggested that testicular production of steroid sulphates can be stimulated by hCG.

INTRODUCTION

In the human testis, many of the sulphate-conjugated precursors of testosterone are present in high concentrations [1]. The testis synthesizes, metabolises and hydrolyses these steroid conjugates and is able to use them as precursors of testosterone *in vitro* [2]. The importance of steroid sulphates in the testis is at present insufficiently known, but they may play an active role in the maintenance of an intratesticular androgen balance [3, 4]. *In vivo*, rapid (approx 2 h) and slow (approx 2–4 days) responses to a single intramuscular injection of 5000 IU of human chorionic gonadotrophin (hCG) have been observed in human testicular secretion of testosterone and some of its precursors, with the slow response prevailing [5, 6]. However, in similar conditions under which the rapid and slow responses to hCG have been seen in unconjugated steroids, no significant increases took place in the testicular secretion of steroid sulphates [7], although it has been shown earlier that administration of 5000 IU of hCG daily for 5 days led to stimulation of the secretion of both unconjugated and sulphated steroids [8]. During oestrogen treatment, the decreases in steroid sulphate concentrations in human testis tissue and in the spermatic vein are very similar to those of unconjugated steroids [3] but the time course and magnitude of the changes suggest that in the case of steroid sulphates they are secondary to those of unconjugated steroids. Under *in vitro* conditions, without added precursors or gonadotrophins, testosterone biosynthesis in human testis tissue is associated with decreases in the testicular concentrations of pregnenolone and dehydroepiandrosterone sulphates, suggesting a role for them as precursors of testosterone formation in these circumstances [4]. In previous work [9] we have demonstrated the ability, *in vitro*, of human testis tissue and Leydig cell preparations to respond to hCG and to 8-bromo-cyclic adenosine 3′,5′-monophosphate (8-bromo-cAMP) stimulation by increased unconjugated testosterone production in short-term (4 h) incubations. In the present work we have investigated the concentrations of steroid sulphates in comparison with those of unconjugated steroids after stimulation of human testicular tissue pieces in short-term incubation with hCG and with 8-bromo-cAMP.

EXPERIMENTAL

Tissue samples

Testis tissue was obtained from three men (56, 72 and 73 years of age) undergoing orchiectomy because of prostatic carcinoma. The patients had not had any endocrine treatment prior to operation. The testes were transported to the laboratory on ice and utilised immediately.

Incubations

In the incubations in which hCG (Pregnyl, Organon, Oss, The Netherlands) was used, testis pieces of about 20 mg were cut and placed in a small petri dish containing sufficient Dulbecco's Modified Medium (Gibco, U.K.) to cover the bottom.

Pieces were selected to be as nearly as possible the same size. Incubations were performed by placing single pieces in 5 ml Dulbecco's Modified Medium containing 0–50 IU hCG per ml. Three vials (plastic, screw top, total capacity 25 ml) for each hCG concentration were placed in a shaking water bath at 35°C for 4 h and the incubations were carried out under an atmosphere of 95% O_2–5% CO_2, replaced every 2 h.

In the incubations in which 8-bromo-cAMP (Sigma, St Louis, MO) was used, the conditions were

similar to those above, but tissue pieces of around 10 mg were used, in an incubation volume of 2 ml, containing 0–1000 μmol 8-bromo-cAMP per l. At the end of the incubation period, the vials were stored at −20°C. The tissue pieces were weighed immediately or after storage at −20°C.

Analytical procedures

The tissue pieces in incubation medium were homogenised by hand in a glass-Teflon homogeniser and the contents transferred to glass extraction tubes. Unconjugated steroids were extracted three times with ethyl ether–ethyl acetate (9:1, v/v), as previously described for serum samples [10], after the addition of a known amount of tritiated testosterone in order to monitor experimental errors.

Methodology for the quantitation of unconjugated and sulphated steroids has been described previously [1, 11], with the exception of 17-hydroxypregnenolone and its sulphate (below). Briefly, unconjugated steroids were analysed as follows: after extraction, the combined organic phases were fractionated on Lipidex-5000™ microcolumns (Packard-Becker, B.V., Chemical Operations, Groningen, The Netherlands), followed by radioimmunoassay of each steroid from the appropriate fraction, using antisera of defined specificity. The recovery of tritiated testosterone was calculated and 85% was the limit of acceptability.

Steroid sulphates were analysed from the water phase of the above-mentioned extractions. They were dried under a nitrogen flow and the residue was dissolved in 3 ml of absolute ethanol. Samples were centrifuged to remove protein precipitates and the supernatant was dried and solvolysed [12] in 3 ml of ethyl acetate that had been equilibrated previously with 2 M H_2SO_4. Before overnight incubation at 37°C, an additional 50 μl of 2 M H_2SO_4 was added to the tubes to assure that the medium remained acidic. After solvolysis, the mixture was neutralised, a known amount of tritiated testosterone was added to monitor experimental errors and the ethyl acetate phase was transferred to another tube, dried, and fractionated on Lipidex-5000™ microcolumns. Chromatography was followed by radioimmunoassay of steroids. Recovery and assay performance after solvolysis were monitored as described for unconjugated steroids.

Antiserum against 17-hydroxypregnenolone

The antigen, 3β,17-dihydroxy-5-pregnen-20-one 3β-hemisuccinate: BSA, was prepared by using the carbodiimide reaction as described previously [13]. Antiserum was raised in rabbits and was used at a 1:4000 dilution, and employing a tritiated ligand (The Radiochemical Centre, Amersham, U.K.). In Table 1 the relative affinity of the antiserum to 3β,17-dihydroxy-5-pregnen-20-one 3β-hemisuccinate: BSA for some steroid ligands is given.

Table 1. The relative affinity (50% binding) of the anti-serum to 17-hydroxypregnenolone for some steroid ligands

Steroid	Relative affinity
17-Hydroxypregnenolone	1.000
3β,17-Dihydroxy-5α-pregnan-20-one	0.405
17-Hydroxyprogesterone	0.214
3β,17-Dihydroxy-5β-pregnan-20-one	0.062
Pregnenolone	0.021
Progesterone	0.005
3β,20α-Dihydroxy-5-pregnen-20-one	0.001
Cholesterol	<0.001
Cortisol	<0.001
Testosterone	<0.001
Dehydroepiandrosterone	<0.001
Androsterone	<0.001
5-Androstene-3β,17β-diol	<0.001
Estradiol	<0.001

Assay of 17-hydroxypregnenolone

The analytical procedure for 17-hydroxypregnenolone was as described above, but in Lipidex-5000™ chromatography the solvent system was light petroleum–chloroform (92:8, v/v). Although the specificity of the antibody against 17-hydroxypregnenolone was checked, it was considered essential to assess whether it could be used to determine 17-hydroxypregnenolone in human testis tissue. In order to do this, we collected 1 ml fractions during the column chromatographic purification of the solvent extracts of both unconjugated and sulphated steroids from approx 500 mg (wet weight) of human testis tissue, and measured individual fractions with the antibody. The chromatographic profiles of 17-hydroxypregnenolone as measured in the fractions of unconjugated (a) and sulphated (b) steroids are presented in Fig. 1. The elution pattern of tritiated 17-hydroxypregnenolone is shown in panel c of Fig. 1. The total fraction volume subsequently used for the measurement of 17-hydroxypregnenolone was collected between 32–48 ml.

The sensitivity of the assay, defined as the mean of the nonspecific water blank values plus 2 SD, was 10 pg per assay tube. The intra-assay coefficient of variation was 8% ($n = 10$) and the total day to day variation was 11% ($n = 10$).

RESULTS

Table 2 shows the stimulatory effect of hCG (1 IU/ml) on the concentrations of steroids and steroid sulphates in human testis tissue *in vitro*. The concentrations are expressed as ng/g and reflect total production by tissue after incubation with and without hCG, using a total of 18 tissue pieces, taken from the same testis.

When human testis tissue from a 72-year old patient was incubated in the presence of various doses of hCG (Fig. 2, a and b), stimulation of the total production of steroids and steroid sulphates was evident at the lowest dose used (0.1 IU/ml). Testosterone production was greatest at 1 and 10 IU hCG per ml (Fig. 2a) whereas production of the majority

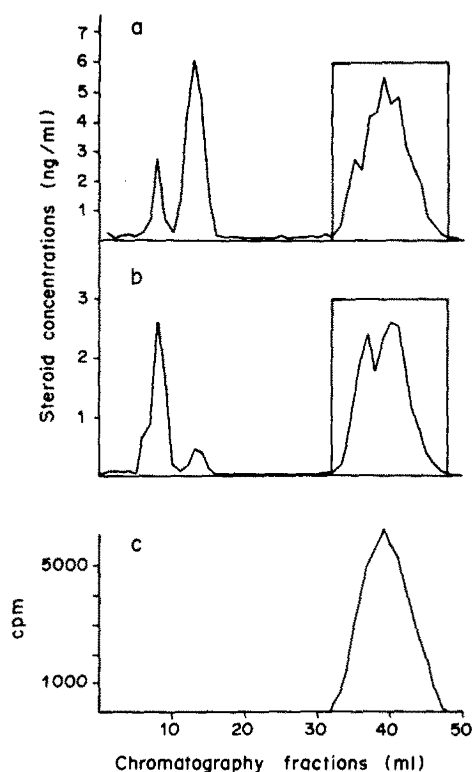


Fig. 1. The chromatographic profiles of 17-hydroxypregnenolone and cross-reacting steroids as measured in the fractions of unconjugated (a) and sulphated (b) steroids of human testis tissue. The elution pattern of tritiated 17-hydroxypregnenolone is shown in panel c. The boxed areas indicate the fraction volumes collected for the radioimmunoassay of 17-hydroxypregnenolone.

of the other unconjugated steroids measured was greatest at the highest dose (50 IU/ml). Total production of steroid sulphates, on the other hand (Fig. 2b), was similar to that of testosterone, in that peak concentrations were seen at 1 or 10 IU hCG per ml, whereas at the highest dose, all steroid sulphate concentrations, with the exception of testosterone sulphate, had returned to that observed after incubation in the absence of hCG.

Stimulation of unconjugated steroids in the presence of various doses of 8-bromo-cAMP (Fig. 3a) was variable and of small magnitude, with the greatest total production being observed at the highest dose used (1000 μmol/l). Under the same conditions, the dose–response curves of the steroid sulphates (Fig. 3b) were all remarkably similar, with a peak appearing at a dose of 1 μmol/l and evidence of further stimulation at 1000 vs 100 μmol/l.

DISCUSSION

In previous work [9] we have demonstrated that human testicular tissue *in vitro* has relatively high basal testosterone production and it responds poorly to gonadotrophic stimulation. Both the basal and stimulated productions of testosterone, on a per gram of tissue basis, show considerable variation between patients (elderly men castrated because of prostatic carcinoma).

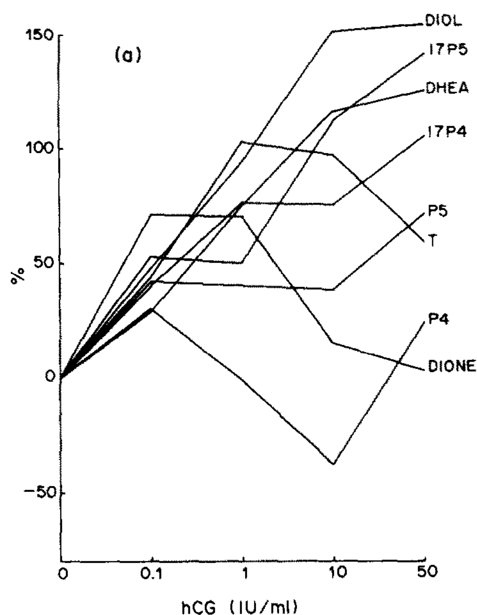
It is clear (Table 2), however, that when human testicular tissue (from a 56-year old patient) was incubated in the presence of hCG, there was significant stimulation of the production of the steroid sulphates involved in the biosynthesis of testosterone, the exception being testosterone sulphate itself. The results compare favourably with those obtained *in vivo* by Laatikainen *et al.* [8], using gas–liquid chromatography and gas chromatography–mass spectrometry to identify and quantify steroids and steroid sulphates in human peripheral and spermatic vein blood. These authors obtained evidence that after 5-day hCG administration (5000 IU per day) to inguinal hernia patients there was increased testicular secretion of monosulphated pregnenolone, dehydroepiandrosterone and 5-androstene-3β,17β-diol, whereas the secretion of testosterone sulphate remained unchanged. 17-Hydroxypregnenolone sulphate was not assayed.

When human testis tissue was incubated with various doses of hCG (Fig. 2, a and b), total testosterone production was maximal at 1 and 10 IU hCG per ml. At the top dose (50 IU hCG/ml), mean testosterone and androstenedione concentrations

Table 2. Steroid and steroid sulphate concentrations (ng/g ± SD) in human testis tissue after incubation alone (Control) or with hCG (1 IU/ml)

	Control	hCG	P*
Progesterone	58 ± 10	92 ± 26	<0.01
17-Hydroxyprogesterone	1449 ± 281	2730 ± 491	<0.001
Pregnenolone	541 ± 216	778 ± 147	<0.02
17-Hydroxypregnenolone	239 ± 45	401 ± 129	<0.01
Dehydroepiandrosterone	214 ± 50	393 ± 120	<0.001
5-Androstene-3β,17β-diol	468 ± 54	1214 ± 371	<0.001
Testosterone	2263 ± 439	4426 ± 1226	<0.001
Pregnenolone sulphate	885 ± 372	1877 ± 969	<0.05
17-Hydroxypregnenolone sulphate	58 ± 24	141 ± 75	<0.05
Dehydroepiandrosterone sulphate	1203 ± 351	1882 ± 531	<0.01
5-Androstene-3β,17β-diol sulphate	789 ± 123	1261 ± 363	<0.01
Testosterone sulphate	1720 ± 1044	1397 ± 588	NS

One patient (56-years old); 9 testis pieces Control, 9 with hCG.
NS = not significant.
*Student's t-test.



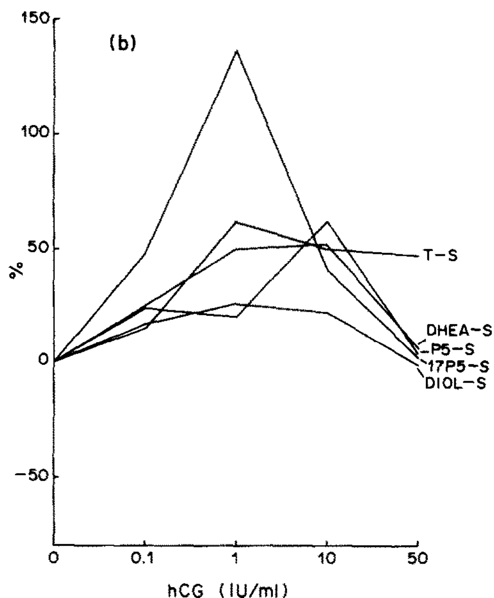
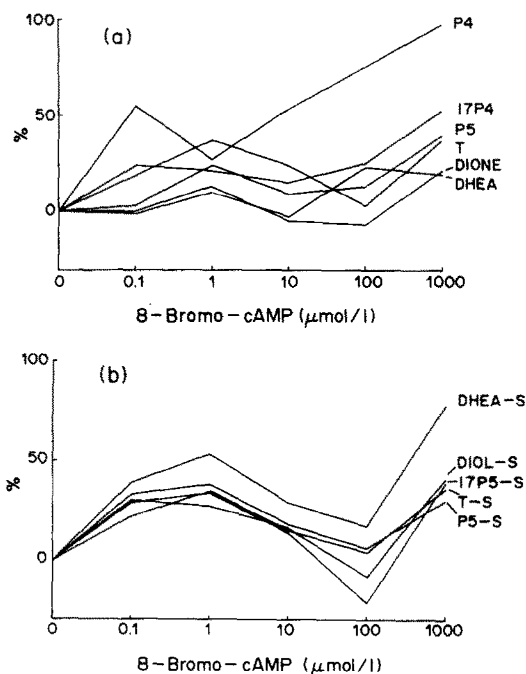


Fig. 2. Percentage changes in the total concentrations of steroids (a) and steroid sulphates (b) after incubation of human testis tissue (72-year old patient) with various doses of hCG. Each point represents the mean of 3 incubations. Steroid and steroid sulphate concentrations were first calculated on a ng/g basis. Abbreviations: P5 = pregnenolone, 17P5 = 17-hydroxypregnenolone, DHEA = dehydroepiandrosterone, DIOL = 5-androstene-3β,17β-diol, P4 = progesterone, 17P4 = 17-hydroxyprogesterone, DIONE = androstenedione, T = testosterone, S = sulphate.



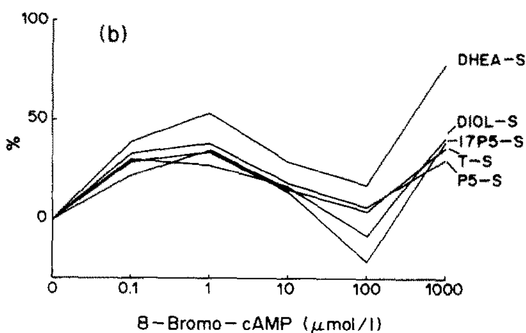


Fig. 3. Percentage changes in the total concentrations of steroids (a) and steroid sulphates (b) after incubation of human testis tissue (73-year old patient) with various doses of 8-bromo-cAMP. Each point represents the mean of 3 incubations. Steroid and steroid sulphate concentrations were first calculated on a ng/g basis. Abbreviations are as for Fig. 2.

were decreased whereas those of the other unconjugated steroids measured were increased.

We have previously reported that when 8-bromo-cAMP (500 mol/l) is used as the stimulating agent in short-term incubation of human testis tissue, a similar low increase in testosterone production is seen as when hCG is used [9]. On the other hand, the production of cAMP after hCG stimulation was increased by 590%.

In the present investigation, pieces of human testis were incubated with 8-bromo-cAMP over a wide concentration range (Fig. 3, a and b). Responses of the unconjugated steroids analysed were moderate, with the greatest values appearing at the highest dose of 8-bromo-cAMP. The concentrations of the steroid sulphates, however, all showed remarkably similar dose–response patterns (Fig. 3b). Although these results were obtained using testis tissue from a single patient, they strongly indicate that it is possible to stimulate the synthesis of steroid sulphates *in vitro* using 8-bromo-cAMP.

We believe that this is the first time that stimulation of human testicular steroid sulphate precursors of testosterone *in vitro* has been reported. In the absence of stimulatory agents or precursors and in comparison with non-incubated human testis tissue, it has previously been reported that in similar incubations as those used in the present study, there was considerable hydrolysis of pregnenolone sulphate, followed by that of dehydroepiandrosterone sulphate. Concentrations of 5-androstene-3β,17β-diol sulphate were unchanged, whereas those of testosterone sulphate were increased [4].

The present data give additional support to the concept that steroid sulphate concentrations are reg-

ulated in close association with the production of unconjugated testicular steroids, especially testosterone. They are retained in the testis tissue, apparently to be used as precursors and reservoirs for the production of unconjugated steroids [4], and their secretion becomes clearly evident only following prolonged stimulation with hCG [8].

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REFERENCES

1. Ruokonen A., Laatikainen T., Laitinen E. A. and Vihko R.: Free and sulfate-conjugated neutral steroids in human testis tissue. *Biochemistry* **11** (1972) 1411–1416.
2. Payne A. H. and Jaffe R. B.: Androgen formation from pregnenolone sulfate by fetal, neonatal, prepubertal and adult human testes. *J. clin. Endocr. Metab.* **40** (1975) 102–107.
3. Leinonen P., Ruokonen A., Kontturi M. and Vihko R.: Effects of estrogen treatment on human testicular unconjugated steroid and steroid sulfate production *in vivo*. *J. clin. Endocr. Metab.* **53** (1981) 569–573.
4. Ruokonen A. and Vihko R.: Quantitative changes of endogenous unconjugated and sulfated steroids in human testis in relation to synthesis of testosterone *in vitro*. Influence of a precursor steroid, dehydroepiandrosterone. *J. Androl.* **4** (1983) 104–107.
5. Saez J. M. and Forest M.: Kinetics of human chorionic gonadotropin-induced steroidogenic response of the human testis. I. Plasma testosterone implications for human chorionic gonadotropin stimulation test. *J. clin. Endocr. Metab.* **49** (1979) 278–283.
6. Martikainen H., Huhtaniemi I. and Vihko R.: Response of peripheral serum sex steroids and some of their precursors to a single injection of hCG in adult men. *Clin. Endocr.* **13** (1980) 157–166.
7. Ruokonen A., Lukkarinen O. and Vihko R.: Secretion of steroid sulfates from human testis and their response to a single intramuscular injection of 5000 IU hCG. *J. steroid. Biochem.* **14** (1981) 1357–1360.
8. Laatikainen T., Laitinen E. A. and Vihko R.: Secretion of free and sulfate-conjugated neutral steroids by the human testis. Effect of administration of human chorionic gonadotropin. *J. clin. Endocr. Metab.* **32** (1971) 59–64.
9. Huhtaniemi I., Bolton N., Leinonen P., Kontturi M. and Vihko R.: Testicular luteinizing hormone receptor content and *in vitro* stimulation of cyclic adenosine 3′,5′-monophosphate and steroid production: a comparison between man and rat. *J. clin. Endocr. Metab.* **55** (1982) 882–889.
10. Hammond G. L., Ruokonen A., Kontturi M., Koskela A. and Vihko R.: The simultaneous radioimmunoassay of seven steroids in human spermatic and peripheral venous blood. *J. clin. Endocr. Metab.* **45** (1977) 16–24.
11. Hammond G. L., Ahonen V. and Vihko R.: The radioimmunoassay of testosterone, 5α-dihydrotestosterone and their precursors in human testis, before and after estrogen treatment. *Int. J. Androl.* (Suppl. 2) (1978) 391–399.
12. Vihko R.: Gas chromatographic-mass spectrometric studies on solvalyzable steroids in human peripheral plasma. *Acta endocr., Copenh.* Suppl. 109 (1966) 1–67.
13. Jänne O. A. and Mäentausta O. K.: Radioimmunoassay of primary and secondary bile acids in serum with specific antisera and ^{125}I-labelled ligands. In *Radioimmunoassay and Related Procedures in Medicine* (Edited by the International Atomic Energy Agency), Vienna, Vol. 2 (1978) pp. 285–293.