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REGULATION OF STEROIDOGENESIS IN TESTIS

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SUMMARY

The endogenous neutral steroids in human, boar and rat testis tissue were investigated by gas–liquid chromatography and gas chromatography–mass spectrometry. In addition, the secretion of these steroids by human testis was investigated under basal conditions as well as during human chorionic gonadotrophin (HCG) administration. In man and rat testis, testosterone was the main steroid hormone present, whereas in boar, 16-androstenes quantitatively occupied the most important position. In man and boar, a large number of 3β-hydroxy-5-ene steroids, to a large extent sulphate-conjugated, were detected. Some of these compounds were secreted by human testis tissue, and their secretion was increased during HCG administration. When considered together with the findings of other authors, these results stress the importance of the 3β-hydroxy-5-ene pathway of testosterone biosynthesis in testicular tissue. Further, they give support to the view that testicular steroidogenesis might be regulated by modulation of the activity of steroid sulphatase in testis. In the evaluation of the regulation of steroidogenesis in testis, species differences must be given serious consideration, as in rat testes no steroid sulphates could be detected.

INTRODUCTION

The quantitatively most important secretion product of testis is testosterone, and it has been amply demonstrated that it is formed by either one of two biosynthetic pathways. The 4-ene pathway proceeds from pregnenolone via 3-keto-4-ene intermediates (pregnenolone → progesterone → 17α-hydroxyprogesterone → androstenedione → testosterone), whereas the conversion of the 3β-hydroxy-5-ene structure to the 3-keto-4-ene structure is the final step in the 5-ene pathway (pregnenolone → 17α-hydroxypregnenolone → dehydroepiandrosterone → 5-androstene-3β,17β-diol → testosterone). The former pathway has been considered to be quantitatively the more important one.

The trophic hormones, luteinizing hormone (LH) and human chorionic gonadotrophin (HCG) increase the production and secretion of testosterone by the testes of a variety of species. There is evidence that their mode of action involves regulation of one or more of the steps between cholesterol and pregnenolone and there is good evidence to suggest that cyclic adenosine monophosphate (cAMP) acts as a mediator of trophic hormone action on steroidogenesis in testis tissue also. The above findings have been presented and discussed in a number of recent review articles[1–4]. The purpose of this paper is to summarize evidence which suggests that in human and boar testis, at least, the formation of testosterone proceeds mainly via 3β-hydroxy-5-ene intermediates and that sulphate conjugates occupy an important position in testicular steroidogenesis.

EXPERIMENTAL

Samples

Human testes were obtained from cadavers shortly after death or in connexion with orchiectomy performed because of prostatic cancer. Human spermatic vein blood was drawn from volunteers undergoing operations for inguinal hernia.

Boar testes were taken immediately after slaughter.

Rat testes were also removed immediately after killing the animals. On certain occasions, interstitial and tubular tissue were separated[5].

All the samples were stored frozen (−20°C) until analyzed.

Analysis of endogenous steroids in testis tissue

A detailed description of the method has been given previously[6]. In short, it is as follows: (i) the tissue is homogenized in acetone–ethanol (1:1 v/v) and chloroform–methanol (1:1 v/v), (ii) the combined dried extract is chromatographed on Sephadex LH-20 in

chloroform–methanol (1 : 1) containing sodium chloride (0·01 M), (iii) fractions of unconjugated, monosulphated and disulphated steroids are collected, (iv) conjugates are cleaved by solvolysis, (v) the free steroids are fractionated by chromatography on silicic acid columns and occasionally on hydroxyalkoxypropyl Sephadex, (vi) the compounds are converted to trimethylsilyl (TMS) and O-methyloxime trimethylsilyl (MO–TMS) derivatives and subjected to gas–liquid chromatography (g.l.c.) on QF-1 and SE-30 columns, (vii) gas chromatography–mass spectrometry (GC–MS) is carried out on an LKB Model 9000 instrument or on a computerized (Varian Spectro System 100 MS) instrument (Varian, Model CH-7).

Analysis of steroids in spermatic venous and peripheral blood plasma was performed as previously described [7]. The method is closely related to that described above for the determination of endogenous testicular steroids and involves extraction of 3–5 ml of blood plasma with acetone/ethanol, separation of unconjugated, monosulphated and disulphated steroids on Sephadex LH-20 in chloroform–methanol containing sodium chloride, solvolysis of conjugates, purification of free steroids on silicic acid and, finally, g.l.c. and GC–MS of the TMS and MO–TMS ether derivatives of the steroids.

The position of the sulphate moiety in steroid diol monosulphates was determined as described by Cronholm [8]. In this method, the steroid monosulphate fraction is acetylated, solvolyzed, purified on silicic acid, the steroids converted to TMS ether derivatives and analyzed by g.l.c. and GC–MS.

RESULTS

Secretion of steroids by human testis

By comparison of steroid concentrations in spermatic and peripheral venous blood the secretion of a number of unconjugated steroids by human testis tissue was demonstrated. Table 1 summarizes the mean concentrations of these steroids in samples from five normal subjects and from three subjects receiving HCG. With respect to the formation of testosterone from pregnenolone, all the intermediates of the 5-ene pathway were secreted and, with the exception of progesterone, all the intermediates of the 4-ene pathway as well. During HCG treatment, traces of progesterone were found in spermatic venous blood. The secretion of all the compounds determined was increased during HCG treatment.

In a series of 11 men [10] testosterone sulphate was present in spermatic venous blood in concentrations of <1–3·9 μg/100 ml. In the same subjects testicular secretion of pregnenolone sulphate and 5-androstene-3β,17β-diol monosulphate was also demonstrated (Table 2). Dehydroepiandrosterone sulphate was not secreted under basal conditions, but its secretion was evident during HCG administration (Table 2). Pregnenolone and 5-androstene-3β,17β-diol monosulphates were excreted in greater than normal amounts in subjects undergoing HCG treatment.

Table 1. Secretion of unconjugated steroids by human testis*

Compound	Group Normal[5]	HCG-treated[3]†
5-Androstene-3β-17α-diol	4·0	9·2
5-Androstene-3β-17β-diol	18·5	78
Dehydroepiandrosterone	2·2	37
Androstenedione	2·5	7·4
Testosterone	74	272
Pregnenolone	4·8	14
17α-Hydroxypregnenolone	3·9	34
17α-Hydroxyprogesterone	6·2	23

* Steroid concentration in spermatic venous plasma (μg/100 ml), from [9].

† HCG (Pregnyl, Organon, Oss, The Netherlands) 5000 U as a daily injection for 5 days. Sample taken on day 5.

Concentrations of free and sulphate-conjugated neutral steroids in human testis tissue

Human testis was found to contain 15 unconjugated neutral steroids, 19 monosulphates and 7 disulphates (Table 3). Table 4 summarizes the quantitatively most important compounds detected and measured [6]. This table readily demonstrates the general trend in our findings that 3β-hydroxy-5-ene steroids and their sulphate conjugates occupy a quantitatively important position among testicular steroids.

Position of the sulphate group in steroid sulphates

It was established in the studies described above that human testis tissue both contains and secretes several steroid diol monosulphates. 5-Androstene-3β,17β-diol monosulphate occupies a very important position among these compounds being the diol monosulphate present in highest concentrations in spermatic venous

Table 2. Secretion of steroid monosulphates by human testis*

Compound	Group Normal[11]	HCG-treated[3]†
Pregnenolone sulphate	380	570
Dehydroepiandrosterone sulphate	110	180
5-Androstene-3β,17β-diol monosulphate	170	690
Testosterone sulphate	1·8	3·3

* The values given are mean percentages of steroid concentrations in spermatic venous blood plasma as compared with peripheral blood plasma. The concentration of testosterone sulphate is given as μg/100 ml. From [9, 10].

† HCG treatment as in Table 1.

Table 3. Neutral steroids identified in human testis tissue employing gas–liquid chromatography–mass spectrometry [6, 11, 12]

Compound	Form of conjugation*
Androsterone	M
Epiandrosterone	M
Dehydroepiandrosterone	F, M
Testosterone	F, M
Androstenedione	F
Epitestosterone	M
5α-Androst-16-en-3α-ol	F, M
5α-Androst-16-en-3β-ol	F, M
5,16-Androstadien-3β-ol	F, M
5α-Androstane-3α,17β-diol	F, M, D
5α-Androstane-3β,17β-diol	F, M, D
5-Androstene-3β,17α-diol	F, M, D
5-Androstene-3β,17β-diol	F, M, D
5-Androstene-3β,16α,17β-triol	M
Pregnenolone	F, M
3β-Hydroxy-17α-5-pregnen-20-one (*iso*pregnenolone)	M
17α-Hydroxypregnenolone	M
Progesterone	F
17α-Hydroxyprogesterone	F
20α-Hydroxy-4-pregnen-3-one	F
5α-Pregnane-3α,20α-diol	M, D
5β-Pregnane-3α,20α-diol	M, D
5-Pregnene-3β,20α-diol	F, M, D

* F = unconjugated steroid, M = monosulphate, D = disulphate.

blood (Table 1) and its unconjugated form being the immediate precursor of testosterone in the 5-ene pathway.

In steroid diol monosulphates either of the hydroxyl groups can be conjugated. Using an acetyl group as a marker for the free hydroxyl group in steroid diol monosulphates, the site of conjugation was determined for the endogenous diols in human testis tissue[12]. It was found that 17β-hydroxy-C_{19} steroid diols were exclusively conjugated at C-3 (Table 5). Similarly, 5-pregnene-3β,20α-diol was conjugated at C-3. In contrast the 17α-hydroxy-C_{19} diols were exclusively conjugated at C-17 (Table 5).

Table 4. The quantitatively most important endogenous neutral steroids in human testis tissue[6]

Compound	Concentration μg/100 g wet weight
Dehydroepiandrosterone sulphate	65
Pregnenolone sulphate	60
Testosterone	43
5-Androstene-3β,17β-diol monosulphate	30
Pregnenolone	27

Table 5. Position of the sulphate group and concentrations of steroid diol monosulphates in human cadaver testis[12]

Compound	Concentration* μg/100 g wet weight
5α-Androstane-3α,17β-diol 3-SO_4	~1·5†
5α-Androstane-3β,17α-diol 17-SO_4	~1·5†
5α-Androstane-3β,17β-diol 3-SO_4	~2·5†
5-Androstene-3β,17α-diol 17-SO_4	42
5-Androstene-3β,17β-diol 3-SO_4	144
5-Pregnene-3β,20α-diol 3-SO_4	43

* Mean concentrations of 3 cadaver testes.

† Exact quantitation was not possible because of disturbing peaks in the g.l.c. analyses.

Steroids in boar and rat testes

In boar testis tissue, 9 unconjugated and 17 monosulphated neutral steroids were detected, whereas no steroid disulphates could be found[13]. A number of 16-androstenes were the principal compounds present, and, as in human testis, several 3β-hydroxy-5-ene steroids were present, also in sulphate-conjugated form (Table 6). The eventual presence of testosterone or its sulphate could not be demonstrated, the level of detection being about 0·5 μg/100 g tissue. Steroid diol monosulphates were conjugated in the same way as in man. C_{19} steroid diols with a 17β-hydroxyl group were conjugated at C-3, whereas the 17α-epimers were conjugated at C-17.

Rat testes displayed an endogenous steroid pattern very different from that in man and boar[14]. Only 3 unconjugated steroids, testosterone (the main component), pregnenolone and 5α-androst-16-en-3β-ol were identified. No sulphate-conjugated steroids were

Table 6. The quantitatively most important endogenous neutral steroids in boar testis tissue[13]

Compound	Concentration* μg/100 g wet weight
5α-Androst-16-en-3β-ol	1830
5α-Androst-16-en-3α-ol sulphate	240
5α-Androst-16-en-3α-ol	160
5α-Androst-16-en-3β-ol sulphate	130
Pregnenolone	115
5α-Androstane-3β,17β-diol monosulphate	90
3β-Hydroxy-5α-pregnan-20-one	70
5-Androstene-3β,17β-diol monosulphate	65
Pregnenolone sulphate	50

* Mean concentrations of 3 boar testes.

detected. The steroids found were quantitated in total testis tissue, in separated interstitial and tubular tissue as well as in cryptorchid testes[14]. The quantitative determinations indicate that the three steroids are synthesized in the interstitial tissue of rat testis.

DISCUSSION

The presence and secretion of 3β-hydroxy-5-ene steroids and their sulphate conjugates by human testis tissue has raised the question of their eventual importance for the endocrine function of the testis. It is especially noteworthy that apart from testosterone these compounds represent the main compounds present in and secreted by human testis tissue. Earlier, the operation of the 5-ene pathway in the testicular biosynthesis of testosterone was amply demonstrated, but its quantitative importance was considered inferior to that of the 4-ene pathway (summarized in Refs. 1 and 3). Quite recently, however, on the basis of incubation studies with human testis, Yanaihara and Troen [15] postulated that the predominant pathway from pregnenolone to testosterone proceeds via pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone and 5-androstene-3β,17β-diol to testosterone.

The main fraction of these 3β-hydroxy-5-ene steroids is present in human and boar testis tissue in sulphate-conjugated form[6, 13]. When considered in conjunction with the results of certain *in vitro* studies[15–17], these findings suggest that certain 3β-hydroxy-5-ene steroid sulphates may be precursors of testosterone. If so, testosterone biosynthesis could be regulated by regulation of the activity of testicular steroid sulphatase. In this regard, Payne *et al.*[18] demonstrated the existence of an excellent correlation between testicular steroid sulphatase and 3β-hydroxysteroid dehydrogenase-isomerase activities and serum testosterone levels. Later it was shown that seminiferous tubules from human testes have the capacity to convert pregnenolone sulphate to testosterone[19].

Working with human testis, Payne[20] found evidence that pregnenolone sulphate, dehydroepiandrosterone sulphate and 5-androstene-3β,17β-diol 3β-yl sulphate are cleaved by the same testicular sulphatase. She further investigated the inhibition of the sulphatase reaction by a number of unconjugated steroids and observed that the kinetics of inhibition are consistent with partial competitive inhibition[20]. Two of the most potent unconjugated inhibitors, 5-pregnene-3β,20α-diol and 5α-androstane-3α,17β-diol are present in human testis tissue[6] and therefore it is possible that the regulation of the release of free steroid precursors of testosterone is effected by regulation of testicular sulphatase by endogenous unconjugated steroids. Using the rat as an experimental animal, Notation and Ungar[21], however, concluded that the regulation of the hydrolysis of steroid 3β-yl sulphates does not constitute a primary mode of action for gonadotrophic hormones. It is to be observed, however, that no steroid sulphates were found in rat testis[14] suggesting that regulation of steroidogenesis in rat testis must be very different from that in man and boar. In the human, HCG administration has a pronounced effect on the testicular metabolism of both unconjugated and sulphated steroids[9].

The conjugation of steroid diol monosulphates in human and boar testes[12] displays a distinct pattern. The 17β-hydroxy-C_{19} steroid diols as well as 5-pregnene-3β,20α-diol are exclusively conjugated at C-3 whereas 17α-hydroxy diols are only conjugated at C-17. The presence of the corresponding disulphates in testis tissue makes it clear that in testis tissue there exists a strict compartmentalization in the formation and metabolism of unconjugated, monosulphated and disulphated steroids. Whether the activity, specificity or distribution of testicular steroid sulphokinase[22] are of importance in the regulation of steroidogenesis in testis remains to be investigated.

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DISCUSSION

Sjövall:

The mode of conjugation corresponds exactly to the one we published for plasma steroids (Cronholm T. (1970) *Steroids*, **14**, 285). What do you think about the origin of circulating 5-androstene-3β,17β-diol 3-sulfate? We have tried to study this by giving ethanol to change the redox state of the liver and see if the androstenediol concentration increases. We have also given [1,1-$^2H_2$] ethanol and found an extensive transfer of deuterium to the 17α-position of androstenediol. These data indicate that most of the 5-androstene-3β,17β-diol 3-sulfate is formed by reduction of dehydroepiandrosterone sulfate in the liver (Cronholm T. and Sjövall J. *Eur. J. Biochem.* **13**, (1970) 124). How much do you think comes from the testes and do you think that the peripheral androstenediol sulfate is taken up by the testes?

Vihko:

I really can't say what the contribution of testis is to androstenediol monosulphate in peripheral blood. I think it or its precursor is adrenal in origin because androstenediol monosulphate is present in much higher concentrations than pregnenolone sulphate in peripheral blood so it obviously has some other source than the testis. A small fraction may be testicular in origin.

Sjövall:

It would be bad also if the testicular production of testosterone were dependent on the redox state of the liver.

Domínguez:

I will report this afternoon some of our recent findings in relation to the effect ACTH on the adrenal steroid sulfatase activity. However, I would like to know if any particular change has been observed on the testicular steroid sulfatase after stimulation with human chorionic gonadotrophin. Actually, the increase of the free steroids, as well as the sulphates, shows stimulation through the pathways following TPNH requiring enzyme systems. In this particular situation, the data you have does not clearly show if the sulfatase activity was actually increased or not, which may be a very important point.

Vihko:

That really is a very important point. We have done some investigations in that respect but they are not so ready that I would like to comment on them at the present moment.

Pasqualini:

In your data, you presented that 17α-androstenediol is exclusively 17-monosulphate and 17β is exclusively 3-monosulphate. Do you have any idea of the possibility of the existence of two different sulfokinases or is it the structure of the steroids that gives this differentiation?

Vihko:

That is a very good question that should be investigated. The sulphokinases of the testis are not well known.

Neher:

I think that this sulfate business has been going on now for at least 15 yr. I wonder what is the significance of the sulfates. You alluded to the possible significance of sulfates as precursors for androgens. You showed also that by human HCG you can increase the sulfate secretion, so one should think there would be a possible significant physiological reaction of these sulfates. Do you think there is a transformation of the sulfates in some other tissue or a physiological function of these sulfates in any other tissues?

Vihko:

That really is a question that has existed maybe for more than 15 yr. I think dihydroepiandrosterone sulphate has been the most investigated compound in this respect because it is the quantitatively most important steroid hormone circulating in blood plasma. As far as I know there are no effects or no phenomena which would indicate a physiological role for this sulphate conjugate. They might have some important task during pregnancy since they are present in very high concentrations in foetal blood. I think this possibility of them being precursors of active steroid hormones is a very important one. This is the first demonstration of some kind of physiological importance for them.

Cooke:

Have you any evidence to demonstrate that the steroid sulfates are formed by conversion of one sulfate to another or do you think that the free steroids are formed first and then sulfated. We did some work with another sulfating gland, the human fetal adrenal gland which sulfates very well, but the main pathways were found to be the free steroid pathways. Once the free steroid has been formed it is then sulfated. (Cooke, Cowan & Taylor, *J. Endocr.* **47** (1970) 295).

Vihko:

On several occasions a direct pathway in the formation of various steroid sulphates has been demonstrated and I think in fetal adrenal, too, there is a direct pathway in the formation of pregnenolone sulphate and dehydroepiandrosterone sulphate.

Cooke:

The direct sulfate pathway has been shown, but I think quantitatively this is a minor pathway. (Perez-Palacios, Perez and Jaffe, *J. clin. Endocr.* **28** (1968) 19).

Vihko:

There are differences of opinion in that matter. We have been able to do studies which we interpret to prove that the sulphated pathway is the more important one (Huhtaniemi, I: *Steroids* **23** (1974) 145–153). In testicular tissue I do not think a direct conversion of one sulphate to another has been demonstrated to date.

Pasqualini:

Concerning the question of Dr. Cooke, I'd like to make some comments. For many years it has been proven in different laboratories, including ours, that in the different human fetal tissues and particularly in the fetal adrenals there is a great capacity for sulfokinase activities but this intensity is different for the different steroids: in general the steroids with a 3β-hydroxy-5-ene structure (e.g.: dehydroepiandrosterone, pregnenolone, 21-hydroxy-pregnenolone, 17α,21-dihydroxy-pregnenolone) are sulfoconjugated in the fetal adrenal in a proportion of 80–90% but the C_{21} sulfate esters such as: cortisol-21 sulfate or corticosterone 21-sulfate are in a lesser proportion of 6–40% (*Excerpta Med. Intl. Congr. Ser.* **219** (1971) 487–495), suggesting the presence of different sulfokinases or that the sulfoconjugation is a function of the steroid structure related to the problem of a "direct metabolism" of a steroid sulfate, this was not only demonstrated for the transformation of pregnenolone sulfate to dehydroepiandrosterone sulfate, as Dr. Vihko mentioned, but also for a direct conversion of the steroid hormone sulfate to the steroid metabolite sulfates (e.g. corticosterone sulfate to different tetrahydrocorticosterone sulfates, (*Acta endocr.* **56** (1967) 308–320)). Finally, I'd like to mention that in a series of studies carried out in the fetal compartments of other animal species such as rat and guinea pig and using different steroid hormones such as: testosterone, cortisol, cortisone, corticosterone, aldosterone, it was found that most of these steroids circulate in the fetal compartment in unconjugated form, which leads to the conclusion that the sulfokinase activity for the steroid hormones during fetal development depends also on the species considered.