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TESTOSTERONE METABOLISM: A NECESSARY STEP FOR ACTIVITY?

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SUMMARY

The 5α-reductase activity and the 3α-hydroxysteroid-dehydrogenase activity of levator ani and skeletal muscles, submaxillary gland, pancreas, kidney, anterior pituitary and prostate collected from adult normal male rats have been analyzed using an "*in vitro*" approach. Labelled testosterone has been used as the substrate. It has been found that the prostate, the anterior pituitary, the submaxillary gland and the pancreas have a rather elevated 5α-reductase activity. The kidney has a limited capacity to form 5α-reduced metabolites of testosterone. The levator ani and skeletal muscles do not seem to possess any 5α-reductase activity. The activity of the 3α-hydroxysteroid-dehydrogenase was found to be totally unrelated to the 5α-reducing capacity of each structure.

INTRODUCTION

It is now generally accepted that in the classical androgen-dependent peripheral structure (prostate, seminal vesicles, preputial gland, sebaceous glands, scrotal skin, epididymus, etc.) testosterone must be transformed into 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT), 5α-androstan-3α, 17β-diol (3α-Diol), and 5α-androstan-3β, 17β-diol (3β-Diol) in order to express fully its activity [1–7]. The conversion of testosterone into these metabolites involves two separate enzymatic processes. First of all, a 5α-reductase transforms testosterone into DHT. Subsequently, two 3-hydroxysteroid-dehydrogenases convert DHT into 3α-Diol and 3β-Diol, respectively.

More recently, it has been demonstrated that also several central structures sensitive to testosterone (hypothalamus, anterior pituitary, etc.) are able to convert the hormone into DHT and 3α-Diol [8]. It has also been shown that castration enhances the ability of the anterior pituitary and of the hypothalamus of male rats to transform testosterone into its 5α-reduced metabolites, while treatment of castrated animals with either testosterone or estradiol significantly diminishes the ability of these structures to metabolize testosterone into its "active" metabolites [8]. Data have also been presented which indicate that, both in the anterior pituitary and in the hypothalamus, the activity of the 5α-reductase is higher in prepubertal than in sexually mature male rats [9], a finding which fits with the hypothesis that the threshold of activation of the central "gonadostat" which controls puberty in male animals changes at time of sexual maturation [10–14]. Finally, Zanisi, Motta and Martini[15] have reported that DHT and 3α-Diol are much more effective than testosterone in suppressing LH release in castrated animals. On the basis of these findings it has been postulated that testosterone exerts its feedback effects via a preliminary transformation into its traditional 5α-reduced "active" metabolites [8, 9, 15].

In the body, there are several other structures which respond to testosterone, even if they are not normally included in the list of "classical" androgen-depending tissues. These non-conventional testosterone-sensitive structures include among others: the kidney[16–19], the skeletal and the levator ani muscles[20, 21], the bone marrow[22], the submaxillary gland[23–28], etc. It has been demonstrated that the cytoplasm of the cells of these structures contains a receptor protein, which binds testosterone and other androgens, and which is similar to that present in the classical androgen-dependent structures [1, 29–31]. However, little information is available on the metabolic processes which occur in the cells of these structures. In particular, it has not been fully clarified whether the transformation of testosterone into its usual 5α-reduced metabolites (DHT, 3α-Diol and 3β-Diol) is a necessary prerequisite for the action of the hormone. The work here to be described has been devoted to the clarification of this point. Fragments of the levator ani and of the skeletal

muscles, of the submaxillary gland and of the kidney taken from normal male rats have been incubated "*in vitro*" in the presence of labelled testosterone. The transformation of the hormone into its 5α-reduced metabolites has been evaluated at the end of the incubation period. Fragments of the prostate and of the anterior pituitary gland have been used as controls. Pancreatic tissue has also been included in this study, because of its histological similarities with the submaxillary gland. Several of the structures considered formed several other metabolites, in addition to DHT and 3α-Diol. The description of these other metabolites is beyond the scope of the present paper and will be the subject of a separate publication (Massa and Martini, in preparation).

MATERIALS AND METHODS

Reagents

All organic solvents were obtained from Merck. They were not further purified before being used. The cold standards of steroids were purchased from Vister, Casatenovo Brianza (Italy). [4^{14}C]-Testosterone was obtained from the Radiochemical Centre, Amersham (England). [4^{14}C]-Dihydrotestosterone was prepared from [4^{14}C]-testosterone by means of an "*in vitro*" incubation with prostatic tissue of adult male rats. The DHT formed was separated by thin layer chromatography in chloroform : acetone-n-heptane 4 : 1 : 3 by vol.

Thin layer chromatographic (TLC) plates were obtained from Merck. X ray films (type T) were purchased from 3M.

Scintillation fluid was prepared dissolving 5 g of PPO and 0·1 g of POPOP in 1 l. of toluene.

"*In vitro*" *incubation*

Sprague-Dawley male rats weighing 200–250 g, kept in an artificially illuminated (14 h of light, 10 h of dark), temperature controlled room, were used. Animals were killed from 9.00 to 11.00 a.m. with a guillotine. Specimens of 15 mg of the tissues to be examined were collected and placed immediately in glass vials containing 70·000 counts/min of [4^{14}C]-testosterone (specific activity: 59·4 mCi/mmol) or [4^{14}C]-dihydrotestosterone (specific activity: 59·4 mCi/mmol) in 2 ml of a Krebs–Ringer bicarbonate buffer solution. Incubation was performed in a Dubnoff metabolic shaker at 37°, for 3 h, under an atmosphere of 95% of oxygen and 5% of carbon dioxide. After 3 h the incubation was stopped by placing the vials in a refrigerator at −20°C.

Extraction, separation and identification of the metabolites

The whole incubation media with the tissues were transferred into a 50 ml separatory funnel, washing the vial with 2 ml of distilled water and then with 5 ml of ether. They were subsequently extracted twice with 20 ml of diethyl ether. The pooled extracts were washed with 5 ml of distilled water, and then transferred into a 50 ml conical flask filtering them through a bed of 8–10 g of anhydrous sodium sulphate. The ether was then evaporated with a rotating evaporator at 40–50°C under vacuum. The dry residue was dissolved twice in 0·25 ml of ether and transferred on a silica gel thin layer plate (size: 20 × 20 cm; thickness 0·25 mm). Thin layer chromatography was run in chloroform : acetone : n-heptane 4 : 1 : 3. In this system testosterone, DHT, 3α-Diol, androstenedione and androstanedione formed during the incubation period are completely separated. However, androsterone is not separated from dihydrotestosterone. Therefore, in the cases in which this metabolite was also formed, the fraction containing DHT and androsterone was recovered separately, heptafluorobutyrated and chromatographed again by means of TLC in benzene : ethyl acetate 9 : 1.

After thin layer chromatography, an X-ray film was placed on the plate and left in contact for 48 h in the dark. After this time the film was developed and the spots that appear were used for the localization of the radioactive metabolites on the TLC plate. The silica gel on which each metabolite is adsorbed was scraped into a different plastic vial. Scintillation fluid was then added and the radioactivity was counted in a Packard Tri-Carb 3375 liquid scintillator. The different metabolites were identified on the basis of their RF's in TLC systems, their retention times in gas liquid chromatography, and by re-crystallization to constant specific activity, as previously described [32].

RESULTS

Two parameters have been selected: (1) the total amounts of 5α-reduced metabolites formed. This figure provides a clear indication of the activity of the 5α-reductase present in the different tissues; and (2) the percentage quantities of 3α-Diol contributing to the total amounts of 5α-reduced metabolites. This index provides a satisfactory estimation of the efficiency of the 3α-hydroxysteroid-dehydrogenase, i.e. of the second enzyme involved in the process of intracellular androgen "activation".

Table 1 shows quite clearly that the prostate of normal male rats is able to convert significant amounts of testosterone into its corresponding 5α-reduced metabolites. It is also apparent that, in the prostate, the contribution of 3α-Diol to the totality of the reduced metabolites formed is only of about 8%. The anterior pituitary is also able to form significant amounts of 5α-reduced metabolites. The activity of the 5α-reductase of the anterior pituitary, however, is only one fifth of

Table 1. Conversion of testosterone to 5α-reduced metabolites (5α-androstan-17β-ol-3-one and 5α-androstan-3α, 17β-diol) by different androgen-dependent tissues of normal male rats*

Tissue†		5α-reduced metabolites pg/mg‡	% of 5α-androstan-3α, 17β-diol
Prostate	[10]	4858·4 ± 495·6	7·80 ± 0·79
Anterior pituitary	[7]	1091·8 ± 84·1	29·50 ± 2·40
Levator ani	[11]	64·4 ± 5·2	N.D.
Skeletal muscle	[5]	78·4 ± 9·2	N.D.
Submaxillary gland	[11]	1247·4 ± 467·4	36·09 ± 3·80
Pancreas	[9]	2081·9 ± 511·4	38·01 ± 2·56
Kidney	[12]	278·6 ± 26·0	41·24 ± 4·45

* Values are means ± S.E.

† Number of experiments performed in parentheses.

‡ Picograms of steroid formed per mg of wet tissue following a 3-h incubation with 160 ng of [4-^{14}C] testosterone (Specific activity: 59·4 mCi/mmol).

that of the prostate. This is confirmatory of the previous evidence of this and other laboratories [8, 9, 33–43]. In the anterior pituitary about 30% of the 5α-reduced metabolites is in the form of the 3α-Diol. The data presented in Table 1 indicate that the levator ani and the skeletal muscles do not convert testosterone into 5α-reduced metabolites. On the contrary, the submaxillary gland seems to have a rather elevated 5α-reductase activity. The converting activity of this gland is quantitatively similar to that of the anterior pituitary. In this structure, as in the anterior pituitary, around 30% of the reduced metabolites is in the form of 3α-Diol. The pancreas is also able to convert testosterone into DHT and 3α-Diol in a fashion very similar to that of the submaxillary gland. Finally, the kidney forms very little amounts of 5α-reduced metabolites. Moreover, this structure seems to be able to transform more than 40% of DHT into 3α-Diol.

The fact that the levator ani and the skeletal muscles form extremely limited amounts of 5α-reduced metabolites prevented the direct evaluation of the efficiency of the 3α-hydroxysteroid-dehydrogenase in these structures. Consequently, an additional experiment was designed in order to investigate this point. Fragments of levator ani and skeletal muscles have been incubated with labelled DHT and the amounts of 3α-Diol formed have been evaluated. It is clear from Table 2 that the levator ani and the skeletal muscles are both able to convert to a considerable extent DHT into 3α-Diol. Consequently, these two structures which do not have a 5α-reductase system, do contain the 3α-hydrosteroid-dehydrogenase.

Table 2. Conversion of dihydrotestosterone (DHT) to 5α-androstan-3α, 17β-diol by the levator ani and the skeletal muscle of normal male rats*

Tissue†		5α-androstan-3α, 17β-diol (pg/mg)‡
Levator ani	[3]	3609·8 ± 122·5
Skeletal muscle	[3]	2966·3 ± 174·2

* Values are means ± S.E.

† Number of experiments performed in parentheses.

‡ Picograms of steroid formed per mg of wet tissue following a 3-h incubation with 160 ng of [4-^{14}C] dihydrotestosterone (Specific activity: 59·4 mCi/mmol).

DISCUSSION

From the data it appears that different androgen-sensitive structures metabolize testosterone in a completely different pattern. Some convert testosterone into 5α-reduced metabolites, while others do not. According to the results here presented, one might subdivide testosterone-depending structures into three categories: (a) structures which form high amounts of 5α-reduced metabolites (e.g., the prostate, the anterior pituitary and the submaxillary gland); (b) structures which form only limited amounts of 5α-reduced metabolites (e.g., the kidney), and (c) structures which do not form 5α-reduced metabolites at all (e.g., the levator ani and the skeletal muscles).

Our observation regarding the absence of the 5α-reductase in the levator ani and the skeletal muscles are similar to previous findings of Gloyna and Wilson [44], of Becker and co-workers[45] and of Krieg, Szalay and Voigt[46]. It is interesting to note that, in agreement with our findings, Becker *et al.*[45] have reported that the human skeletel muscle is able to convert DHT into 3α- and 3β-androstandiols "*in vivo*". The finding that the kidney converts only limited amounts of testosterone into DHT and forms rather large amounts of 3α-Diol is also supported by literature data [19, 31]. However, some conflicting results have also appeared. Verhoeven and De Moor[17] have reported that purified nuclear fractions of kidneys of male and female rats form elevated amounts of DHT; however, only the male kidney is able to further metabolize DHT to 3α-Diol. The discrepancy between the data here reported and those of Verhoeven and De Moor[17] may probably be explained by methodological differences. In our study, fragments of kidney were incubated, while, in the other study, purified nuclear fractions have been used.

In agreement with our data, Booth[47] has recently obtained "*in vivo*" evidence for the formation of DHT in the submaxillary gland of the boar after the administration of labelled testosterone, and Coffey[48] has observed that submaxillary gland homogenates of male mice convert testosterone to 3α-Diol. However, in his experiments the presence of a NADPH generating

system was necessary. This co-factor was not found to be essential in our study. The finding that the pancreas is able to metabolize testosterone in a fashion similar to the submaxillary gland is totally new, and deserves further consideration since this structure is usually not believed to be androgen dependent, or to show a sexual dimorphism. The reasons for the presence of such an elevated 5α-reductase activity in this structure are obscure at the present time.

An important observation which emerges from the data here presented is that the two enzymatic systems which convert testosterone into DHT and 3α-Diol (the 5α-reductase, and the 3α-hydroxysteroid-dehydrogenase) are not linked together. First of all, it has been found that the 3α-hydroxysteroid-dehydrogenase activity exists in all structures examined, independently from whether they contain the 5α-reductase or not. Moreover, in those structures in which the 5α-reductase is present, the activity of the 3α-hydroxysteroid-dehydrogenase seems to be totally unrelated to that of the first enzyme. For example, the prostate which has very high levels of 5α-reductase has a low 3α-hydroxysteroid-dehydrogenase activity. On the contrary, structures like the anterior pituitary, the submaxillary gland, the pancreas and the kidney which all have a 5α-reducing capacity lower than that of the prostate, seem to have more elevated 3α-hydroxysteroid-dehydrogenase activity.

It is not within the scope of the present publication to discuss in full detail the physiological role(s) played by the 3α-Diol, nor the possible significance of its differential formation in the different structures. It has been pointed out that the formation of the androstandiols might represent an inactivation process, necessary for protecting certain structures from the presence of too high levels of "effective" androgens [17, 49, 50]. However, several recent findings indicate that both the 3α- and the 3β-Diol exert significant biological effects in many androgen-dependent systems [6, 7, 15, 51–56]. These observations certainly support the view that androstandiols might play an important physiological role for the full expression of testosterone (or DHT) activity.

The data here presented may bring to the conclusion that the theory that testosterone exerts its activity on its depending structures only via the conversion to DHT and other 5α-reduced metabolites is probably not totally correct. The data have indeed emphasized the fact that structures certainly sensitive to testosterone like the levator ani and the skeletal muscles do not possess a 5α-reductase system. It is consequently possible that testosterone acts as such on these structures. Obviously, one might suggest that the effects observed in these structures following the "*in vivo*" administration of testosterone might be due to the arrival on their androgen receptors of DHT formed in other structures of the body. However, circulating levels of DHT are normally too low as to make this hypothesis a rather remote one.

There is a practical final consideration which emerges from the data here presented, i.e. that efforts to dissociate the androgenic from the so-called myotropic-anabolic activity might eventually be successful. The present results clearly underline the fact that one steroid may exert its androgenic and anabolic effects via totally divergent mechanisms.

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DISCUSSION

Müller:

Dr. Martini, I have some difficulties comprehending the whole physiological implication of your data which actually show that a hormone suppresses its own activators. What could be the possible role of testosterone inhibiting its own activator in its negative feedback effect on LH secretion?

Martini:

As I said, not only testosterone suppresses the 5α-reductase activity of the anterior pituitary, but also estrogens have the same effect. In agreement with our findings, a recent paper by Lee, Bird and Clark (*Steroids*, 1973, **22**, 677) has beautifully shown that if you incubate prostatic tissue in the presence of testosterone and estrogens, testosterone is no longer converted into DHT. I believe that the activation of the 5α-reductase brought about by castration is a sign of "total" activation of the pituitary gland. Following castration you get an increase in gonadotropin secretion, and, probably, the 5α-reductase is present only in the gonadotrophic cells of the anterior pituitary. If this is so, following castration you have a tremendous increase in the number and activity of these cells and probably the whole enzymatic apparatus of these cells (including the 5α-reductase) is activated. When you treat the animals either with testosterone or with estrogen, you bring back to normal the activity of these cells. The 5α-reductase, as a consequence, will be reduced.

Kuss:

May I ask Dr. Martini if, for example, progesterone is also converted to 5α-metabolites and if substrate specificity is needed for these regulation phenomena.

Martini:

Progesterone is a substrate of the 5α-reductase as well as testosterone. This has been shown by Drs. Massa, Stupnicka and myself (*Excerpta Medica· Int. Congr. Series*, 1972, 256, Abstract n. 293). Progesterone is even a better substrate of the 5α-reductase of the anterior pituitary and the hypothalamus than testosterone itself. If you take the pituitaries of male and female rats, and you test their ability to convert either testosterone into DHT or progesterone into the corresponding 5α-reduced metabolite, you will find that the pituitary of the female is much more active in making the conversion than the pituitary of the male. It is possible that the 5α-reduced metabolites of progesterone are important for pituitary function in female animals. As you all know, progesterone has a double-faced activity, because it may either activate or block ovulation. It is our feeling that this may be explained by the formation of two types of metabolites: one metabolite being the inhibitor, the other being the activator.

Munck:

Dr. Martini, you mentioned that in the feedback inhibition studies you carried out, you used the free alcohols. Would it make any difference if you used the propionates. You assumed that the feedback effect of testosterone on FSH was probably mediated through conversion to estradiol. Are there any cases where testosterone works as such, without conversion?

Martini:

With regard to your first point, we used the free alcohols only because the propionates of 3α-diol and DHT were not

available. We have not tested the propionate but I don't think there will be any difference. The propionate is a long-acting form, while the free alcohol is a short-lived compound. In answer to your second question, I don't assume the conversion of testosterone to estradiol, I just suggested this as a possibility. It is also possible that testosterone acts as such on the control of FSH.

Breuer:

I am very much interested in your statement that you said that the LH secretion is regulated by 5α-DHT, whereas FSH may be regulated by testosterone. It's difficult to see how this could act because as far as I know, this goes via the releasing hormone and I wonder whether you could explain the different actions of the different hormones where there is only one releasing hormone. I have a second question. I think these studies by Naftolin on the aromatization are very impressive and the rate of aromatization is in the order of 1 %. Would you think that in the presence of high amounts of testosterone, estradiol could become effective at the hypothalamic and hypophyseal level?

Martini:

With regard to the first question, we must go to philosophy first. You must consider whether the feedback effect of steroids takes place in the anterior pituitary or in the hypothalamus. We have been firm believers for a long time that the major site of feedback effect of sex steroids was the hypothalamus, and if you still assume that the feedback of sex steroids takes place in the hypothalamus, the data cannot be reconciled with the existence of one single hypothalamic controller of gonadotropin secretion. However, we have plenty of biological data that prove that it is still possible to look for an FSH-RH separated from LH-RH and different from the decapeptide isolated and purified by Schally and his co-workers. But it seems to me that the pendulum of the "sex centre" is swinging toward the pituitary right now. Our own data, for instance, show that the 5α-reductase activity is much higher in the anterior pituitary than in the hypothalamus. When you castrate the animals, you have a very significant increase of 5α-reductase activity in the anterior pituitary while you have a very limited increase in the hypothalamus. So, biochemical data from our laboratory indicate that the pituitary might be at least one site of the feedback activity of testosterone. If this is the case, it does not appear difficult to postulate two separate mechanisms for the feedback control of the two gonadotrophins.

Let me come now to the question of aromatization into estrogens. I think that I briefly mentioned in my presentation that the effect on FSH might be due either to testosterone as such, or to its conversion into estrogens. This occurs both in the hypothalamus and in the anterior pituitary.

Gurpide:

Could the effect of circulating 3α-diol, which seems to be present in very high concentrations at least in female rats, be as significant as the action of the metabolite produced locally, in the hypothalamus or the pituitary?

Martini:

Our experiments have been done in castrated rats, so we assume that there was no 3α-diol circulating in the animals.

Pasqualini:

In one slide you presented some unknown metabolite. Do you have any idea of the structure and the percentage of this unknown metabolite(s) under the different conditions that you study?

Martini:

I think I will give this question to Dr. Massa who may have more information on the more polar metabolites. However, I want to add that the purpose of the work was to study the formation of 5α-reduced metabolites and not to isolate unidentified metabolites which are formed in trace amounts.

Massa:

We have at least 5 or 6 metabolites in very small amounts and in our chromatographic system they ran together. We didn't make any attempt to identify them. We think they are highly hydroxylated compounds but we can't say anything more.

Korenman:

This seems like the most reasonable explanation for the desensitization of the hypothalamic-pituitary axis during puberty. Have you studied the question of reductase activity in puberty.

Martini:

Yes, we—i.e. Dr. Massa has done it. It is very interesting that in all structures we considered (anterior pituitary, hypothalamus, amygdala, the cerebral cortex) the 5α-reductase activity is much higher at birth than it is in adulthood. What is very interesting is that in mature animals the cortex loses totally the ability of converting testosterone into DHT, the amygdala retains some of this activity. If you study carefully day-by-day, the 5α-reducing activity of the anterior pituitary and the hypothalamus, you will find that there are two subsequent drops which correspond exactly with two crucial phases of the sexual maturation of the male animal (Massa, Stupnicka, Villa and Martini, 53*rd Meeting of the Endocrine Society*, 1971, p. A229). We believe these data have provided a biochemical explanation for the theory which suggests that, at puberty, there is a change in the sensitivity of the hypothalamic "gonadostat" to sex steroids. Our data indicate that androgens are better utilized before puberty than after puberty in the central structures.

Lindner:

May I comment on the question whether there exists one or more releasing hormone(s) for the gonadotrophins? You express the belief that the major site of steroid feedback is the hypothalamus and, as a consequence, feel that one must postulate more than one releasing hormone. We have recently obtained a potent and highly specific antiserum to the decapeptide described by Schally and by Guillemin. With this antibody we blocked *both* LH and FSH release in the intact pro-oestrous rat and in castrated female rats (*Biochem. Biophys. Res. Commun.* **55**, (1973) 616 and 623). It thus looks fairly likely that there may really be only one releasing hormone. But this doesn't exclude the possibility that the steroids modulate the effect of the releasing hormone on the pituitary and determine whether the response of the pituitary will be predominantly LH or FSH release, and I think there is some evidence in this direction. Would you like to comment on this?

Martini:

Yes, I would. Let me take the easiest part first. There is no doubt that steroids modulate the effect of the synthetic decapeptide at the anterior pituitary level. Recent, unpublished data from my laboratory indicate that estrogens potentiate the effect of the decapeptide as a releaser of both LH and FSH. If you add progesterone to estrogens, the effectiveness of LH-RH becomes lower. Testosterone, on the

contrary, when given to castrated male animals will facilitate the release of FSH under the influence of LH. LH is less responsive in the presence of testosterone.

With regard to your first comment, i.e. that with the anti-LH-RH antiserum you can block the surge of LH and FSH which is present at pro-estrus in the rat, I agree that this indicates that you have blocked the hypothalamic stimulator which brings about the pro-estrus increase of LH and FSH. I also believe that what brings about the increase of LH and FSH at the time of pro-estrus is the decapeptide. However, it is still possible that a factor different from the decapeptide might control FSH secretion outside the pro-estrus day.

Bertrand:

I have one comment to Prof. Martini. When hypothalamus of immature rats were incubated with testosterone, Dr. Loras showed that in the incubation there was a greater quantity of 4-androstenedione formed than of DHT which suggests that 4-androstenedione could be aromatized in the hypothalamus.