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Review

Epitestosterone

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Abstract

Epitestosterone has been identified as a natural component of biological fluids of several mammals including man. For a long time it was believed that it is a metabolite without any hormonal activity and without any marked relationship to the hormonal state in health and disease. Neither the biosynthetic pathway nor the site of its formation in man have been unequivocally confirmed to date. It apparently parallels the formation of testosterone (T), but on the other hand its concentration is not influenced by exogenous administration of testosterone. This fact creates the basis of the present doping control of testosterone abuse. In 1989 an observation was presented in a dermatological study that epitestosterone exerts an effect counteracting the action of testosterone on flank organ of Syrian hamster. Further studies showed that a complex action consisting of competitive binding of epitestosterone to androgen receptor, of inhibition of testosterone biosynthesis and its reduction to dihydrotestosterone and of antigonadotropic activity could be demonstrated in rat, mice and human tissues. It can be presumed that epitestosterone as a natural hormone can contribute to the regulation of such androgen dependent events as, e.g. the control of prostate growth or body hair distribution.

Keywords: Epitestosterone; Androgens; Biosynthesis; Metabolism; Doping

Contents

1. Introduction

Epitestosterone (17α-hydroxy-4-androsten-3-one) is a naturally occurring epimer of testosterone (T). Clark and Kochakian [15] reported it for the first time in 1947 as an androgen metabolite on incubation with rabbit liver slices. However, the question regarding its origin and potential physiological role are not yet fully answered.

2. Occurrence of epitestosterone

Epitestosterone was recognized as a normal constituent of mares' follicular fluid and bovine testes (see [24]). Similarly, follicular fluid from preovulatory follicles in women

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with stimulated cycles contains high concentration of epitestosterone [20]. Epitestosterone accumulation has been demonstrated in mammary cyst fluid [7] and in human prostate [35].

Curiously, epitestosterone is also a naturally occurring fytosteroid, in the pollen of pine *Pinus silvestris* [56].

Epitestosterone is formed from testosterone and androstenedione in vitro by rabbit liver and kidney, dog liver, mare's ovary, ox and sheep blood, guinea pig liver, kidney, ovary and testes but not by adrenal slices. Furthermore, human whole venous blood, human adrenals and human sclerocystic ovary produce epitestosterone, probably from testosterone and/or androstenedione in vitro (see [2,9]). However, epitestosterone is only a minor metabolite. Unique in its ability to produce epitestosterone from testosterone or androstenedione is the mouse kidney, especially its cytosol subcellular fraction with NADH as cofactor: the yield of epitestosterone reaches 70%. It is of interest that the mouse liver did not produce epitestosterone [2].

In the human urine the excretion of epitestosterone glucuronide and sulfate was first reported in the mid-1960s [11,42]. In the human, epitestosterone is excreted in the urine mainly as glucuronide [21], which has been reported to increase after intravenous administration of a very large amount of testosterone or after stimulation by ACTH or hCG [67,68]. On the other hand, Dehenin and Matsumoto [18] have demonstrated a dramatic decrease of urinary epitestosterone glucuronide and sulfate after long-term testosterone administration to normal men. The relative occurrence of free sulfated and glucuronized epitestosterone in male and female urine is comparable with those of testosterone fractions [26,66].

Urinary excretion of epitestosterone is slightly lower than that of testosterone, being 200–500 nmol per day in males and 80–500 nmol per day in females [8,21,28,30,37,43,46,47]. Plasma concentrations of epitestosterone [8,29,32,34,45] are age dependent and approximate an average of 2.5 nmol/l and in adult men and 1.2 nmol/l in women [32] (Fig. 1). Extensive studies have been done by Finnish investigators [44,55], who identified and quantified free and sulfoconjugated neutral steroids including epitestosterone in testes and in spermatic venous blood. Though the production rate of epitestosterone is only 3% that of testosterone, its excretion rate is about 1/3 that of testosterone in adult men [73].

Epitestosterone and testosterone, free and sulfoconjugated as well as 5-androstene-3β,17α-diol and its 17β-epimer, have been analyzed in peripheral and spermatic venous plasma of patients with varicocele [17] by gas chromatography/mass spectrometry with stable isotope dilution. All these C-19-steroids are secreted by the testes as evidenced by the significant concentration gradients between peripheral and spermatic venous plasma. Half of the daily epitestosterone production is ascribed to the testes and roughly 70% of epitestosterone sulfate is also of testicular origin.

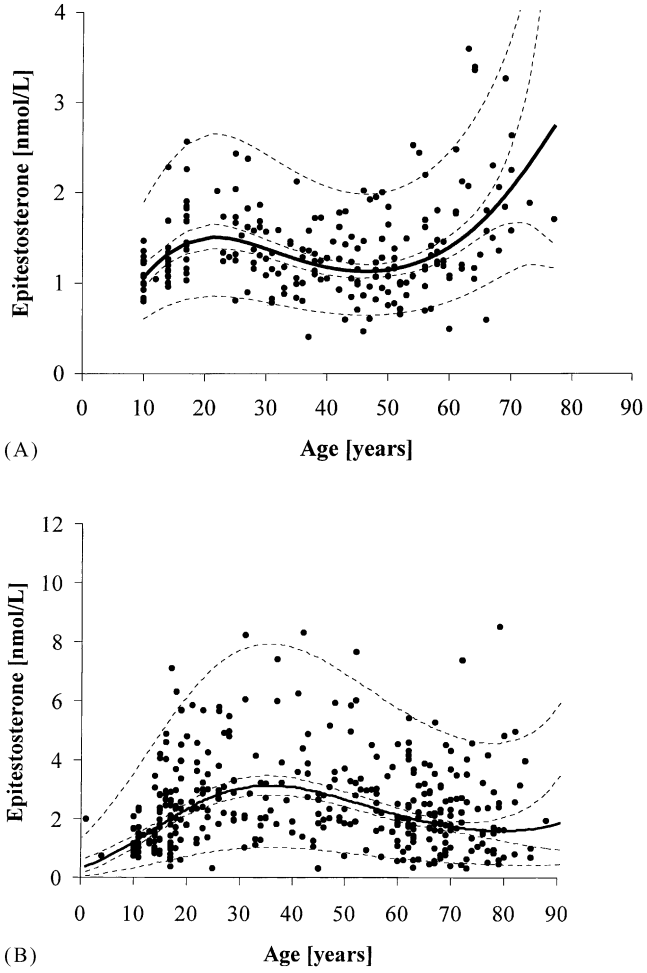

Fig. 1. The age dependencies of epitestosterone concentration in serum of 211 women aged 10–77 years (A) and 386 men aged 1–91 years (B) [32].

3. Epitestosterone in human under various conditions

For the determination of epitestosterone in human urine fluorimetric methods [36,71], gas chromatography [21], gas chromatography/mass spectrometry [22,43,57] and radioimmunoassay [8] were elaborated. The latter method was also applied to the determination of epitestosterone in plasma. Plasma concentrations were first reported by Bílek et al. [8]. Recent data on age dependent plasma concentration of epitestosterone were reported by Lapčík et al. [45] and Havlíková et al. [32] as shown in Fig. 2. For doping control determination of epitestosterone in hair was proposed [14,19,27].

3.1. Urinary excretion

Epitestosterone excretion was increased in many hirsute women [21,28]. However, epitestosterone concentration in urine, unlike that of testosterone, showed no correlation with the virilization of the patients. Subnormal amounts of epitestosterone were excreted by male patients with

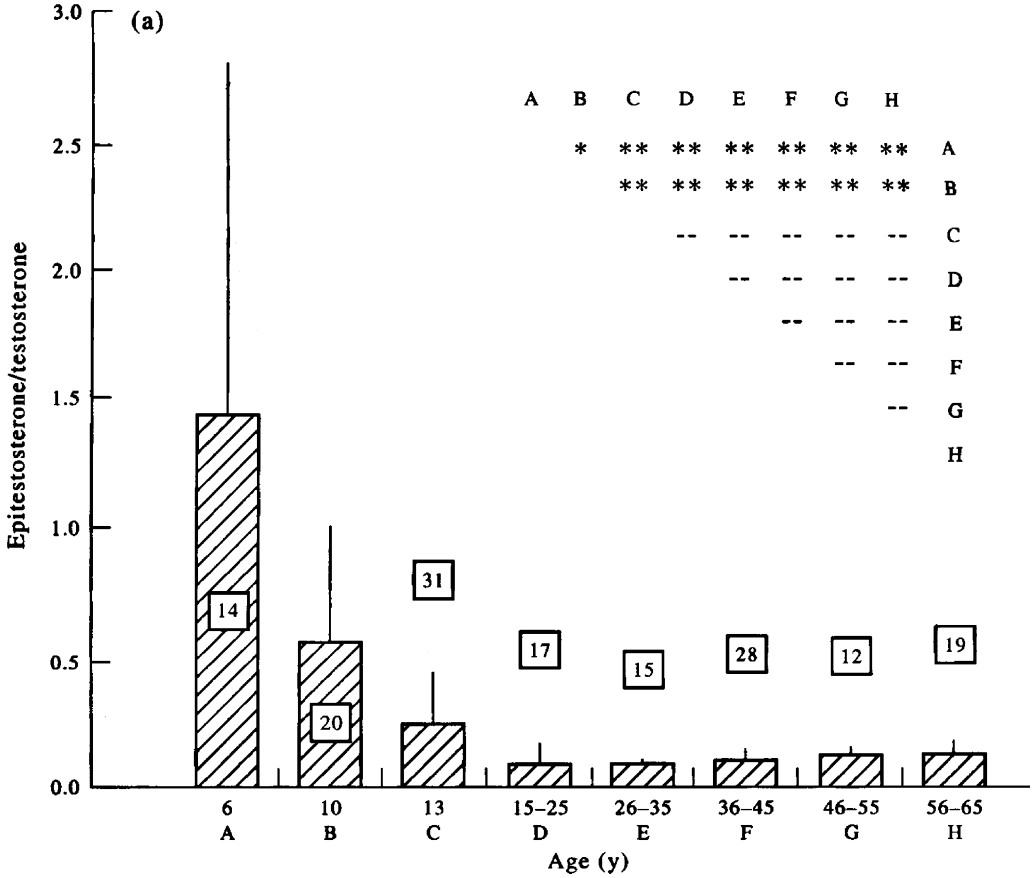

Fig. 2. The ratio of plasma concentrations of epitestosterone to testosterone during the life span in males according to Lapčík et al. [45]. Mean ± S.E.M., the number of cases (n) is given in squares, (*) $P < 0.05$, (**) $P < 0.001$.

hypogonadism [37]. Its excretion was increased by hCG administration, slightly less than that of testosterone [37,46]. In the excretion of epitestosterone in prepubertal children there is neither obvious difference between boys and girls, nor any apparent relation to skeletal age [30]. The excretion increases in boys after puberty and reaches the maximum between 20 and 30 years [37,39] and later, in contrast with a slight decline of testosterone, remains nearly constant to the sixth decade of life. It decreases to nearly prepubertal level in senescence [39]. In normal females the highest epitestosterone excretion was observed on the 22nd and 23rd days of menstrual cycle [47]. There are unique reports on individuals with reduced or practically absent excretion of epitestosterone without any concomitant clinical symptoms [50,52]. In a group of men with benign hypertrophy of prostate in the age of 61–70 years a subnormal excretion of epitestosterone 24.5 ± 12.8 μg per day compared with that of age-matched healthy men 42.8 ± 14.3 μg per day was observed [39].

3.2. Epitestosterone in blood circulation

Plasma values of epitestosterone during the life span were measured by Lapčík et al. [45] and Havlíková et al. [32]. In young boys before puberty, antiandrogenic epitestosterone prevails over testosterone, in adults a striking decline of the ratio epitestosterone:testosterone (E:T) can be observed. The ratio of epitestosterone/testosterone in blood circulation is high in childhood, epitestosterone being more abundant than testosterone before the age of 10 years. Later on, testosterone prevails. The serum concentration of epitestosterone in women exhibits a peak with maximum around 20 years of age followed by continuous decline up to menopause and then a pronounced increase in postmenopausal women (Fig. 1A). In men, serum concentrations of epitestosterone exhibited a peak with significant maximum around 35 years of age followed by continuous decrease later (Fig. 1B). Both in men and women, a pronounced decrease of the epitestosterone:testosterone ratio during puberty and adolescence finished after the onset of puberty on values of between 0.1 and 0.2 in men (Fig. 2) and 0.7–0.8 in women [32,45].

3.3. Tissue concentration

Tissue concentration of epitestosterone has been measured in human testes [55] and hyperplastic prostates [65]. In hyperplastic prostate the epitestosterone concentration (mean 58.4 ± 40.4 S.D., range 14.0–144.0 fmol/mg protein)

is comparable to that of androstenedione, is about double the content of testosterone and approximately half the content of dihydrotestosterone [65]. The prostatic tissue from the patients treated with finasteride for 8 weeks showed a significant decrease not only in dihydrotestosterone content but also in androstenedione and epitestosterone concentration, whereas the concentration of testosterone increases significantly [35].

4. Epitestosterone as a tool for antidoping control

Epitestosterone in the urine has attracted the attention as a reference substance in the doping control of testosterone abuse [22]. The nearly constant ratio of urinary testosterone to epitestosterone ($1.1 - 1.5 \pm 1.0$, range 0.03–4.9) in adults (see [43]) became the basis of the method of detection of exogenously administered testosterone, since epitestosterone does not originate from testosterone in significant amounts in the human. The maximum permissible testosterone to epitestosterone arbitrary ratio in the urine of normal men not suspected for doping has the critical value of 6. The International Olympic Committee adopted this ratio for its accredited laboratories as an arbitrary critical value as the sole test for illicit testosterone self-administration, after the studies done by Donike et al. [22]. Some improvements regarding the pharmacokinetics and dynamics of testosterone epimers and their metabolites have been suggested [18]. The testing is, however, valid only under the assumption that the clearance of both epimers is similar, that testosterone administered exogenously is really not metabolised to epitestosterone, and that the ratio of both epimers is not influenced by racial or individual variations. Some exceptions have recently been observed [16,50,52] in individuals with very low epitestosterone excretion, as have racial differences, e.g. between Japanese (T:E 1.99) and Ainu (T:E 2.77) [53]. The testosterone:epitestosterone ratio is also influenced by intake of various external compounds, e.g. by high doses of alcohol [38,70], more in females than in males. Andostenedione administration increases epitestosterone excretion while decreasing that of its putative precursor [13]. Dehydroepiandrosterone supplementation can increase the testosterone:epitestosterone ratio [10].

Since introduction of the testosterone:epitestosterone ratio in doping analysis, the parameters that may or may not influence this ratio and possibly lead to false positive results have been intensively debated [19,70]. The relevant analytical aspects of the testosterone:epitestosterone ratio, potential parameters of endogenous or exogenous origin, as well as some alternative methods for determining testosterone abuse, such as the urinary testosterone/LH ratios, gas chromatography/combustion isotope ratio, mass spectrometry, hair analysis [14,19,27] and high-performance liquid chromatography/mass spectrometry, have been taken in account to eliminate false positive findings in detection of testosterone abuse by athletes.

5. Origin and biosynthesis

In anima1 species (such as rabbit, guinea pig or mouse), which dispose high activity of 17α-hydroxysteroid oxidoreductase, a substantial proportion of epitestosterone originates from simple interconversion of testosterone to androstenedione and thereafter to epitestosterone. The interconversion is not only species but also organ specific, e.g. mouse kidney but not liver produces epitestosterone from androgen precursors [2]. No interconversion of testosterone and epitestosterone was observed in the testes of bulls, rabbits or rats, in spite of the fact, that the testes is a source of endogenous epitestosterone in these species [61]. It has been demonstrated [48] that in bovine castrate male 37% conversion of testosterone to epitestosterone probably takes place in the blood and liver. The content of testosterone and epitestosterone in urine of a man treated daily with injection of 100 mg androstenedione [11] indicated that epitestosterone in the urine was derived at least in a small part from androstenedione. However, the probability of glandular origin of epitestosterone by an alternative route has also been suggested. The amount of epitestosterone formed from androstenedione is obviously species-specific, depending on the activity of 17α-hydroxysteroid oxidoreductase, not identical with 17β-hydroxysteroid oxidoreductase.

In the human the interconversion of testosterone and epitestosterone is negligible, if any [25]. Wilson and Lipsett [73] have shown unequivocally by experiments with labelled testosterone that epitestosterone in the humans does not originate from testosterone. Thijssen et al. [69] administered ^{14}C androstenedione or ^{14}C testosterone to men and studied ^{14}C content of urinary epitestosterone, concluding that the maximal conversion of androstenedione to urinary epitestosterone was 0.053 ± 0.015% and of testosterone 0.027%. Thus peripheral conversion of androstenedione and testosterone to urinary epitestosterone can account for less than 2 μg/24 h, or under 5% of the total urinary excretion of epitestosterone. The exact amounts of epitestosterone secreted by the testes, adrenal gland, and ovaries are not known; it seems that at least some of it is secreted by adrenal cortex.

The secretion of epitestosterone by the human testes was definitively proved [17] by the determination of the concentration gradient between peripheral vein and spermatic vein plasma level. This secretion was suspected on the basis of previous work demonstrating an increased urinary excretion of epitestosterone glucuronide upon stimulation of Leydig cell function by hCG [67,73]. The decline of epitestosterone excretion, which was observed after chronic testosterone administration [18], was also directly related to impaired Leydig cell stimulation by decreased LH secretion consecutive to the negative feedback exerted by the exogenous testosterone. At least a half of the daily epitestosterone production in males is of testicular origin. A part is probably due to the adrenal gland, since ACTH significantly increases the urinary epitestosterone glucuronide excretion in normal men [67]. This finding is in contrast with other observations.

Kicman et al. [40], using ACTH stimulation of the adrenals of eugonadal and hypogonadal men, have shown that the adrenal contribution of epitestosterone production is relatively modest. Following adrenal stimulation epitestosterone glucuronide in urine increased in hypogonadal men but this increase was masked in eugonadal men because of testicular production of epitestosterone. The mean basal plasma epitestosterone concentration of eugonadal men was twice as high as that of hypogonadal men (1.32 ± 0.08 nmol/l versus 0.68 ± 0.04 nmol/l). In eugonadal men after ACTH stimulation plasma and urinary epitestosterone remained unchanged in contrast to testosterone, the concentration of which decreased in plasma and urine in response to ACTH.

The secretion of epitestosterone sulfate was evidenced in the human testes and the sulfoconjugation of epitestosterone occurred also peripherally in the liver, with 2% overall conversion rate [25].

Studying the 16-ene-synthetase reaction in human testicular homogenates Weusten et al. [72] adduced the evidence for the hypothesis that 5,16-androstadien-3β-ol is synthesised from pregnenolone in a single step, not requiring any separate intermediates, and that such synthesis is always accompanied by co-synthesis of satellite 5-androstene-3β,17α-diol. Pregnenolone binds to the iron atom of cytochrom P-450 of the synthase via its carbonyl at C-20. The steroid nucleus is bound to porphyrin nucleus via hydrophobic interactions while the hydroxyl at C-3 is bound via electrostatic interactions to the propionyl side chain of the porphyrin nucleus. 5,16-Androstadien-3β-ol is synthesized via an elimination reaction that is initiated by a nucleophilic attack of the enzyme at the 16α-hydrogen

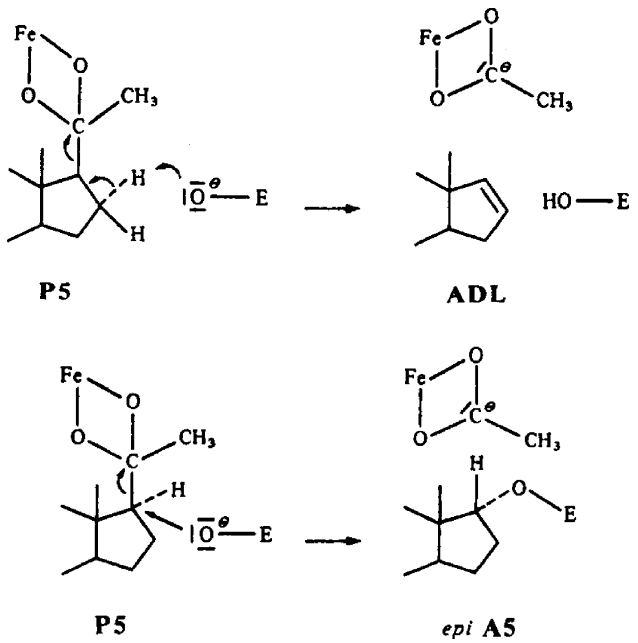

Fig. 3. Mechanism of 5-androsten-3β,17α-diol formation from pregnenolone as a competitive reaction to the synthesis of 16-en steroids according to Weusten et al. [72]. P5, pregnenolone; ADL, 5,16-androstadien-3β-ol; epi A5, 5-androsten-3β,17α-diol; E, enzyme.

of pregnenolone. The synthesis of 5-androsten-3β,17α-diol proceeds via a competing mechanism, i.e. via a nucleophilic substitution that is initiated by a nucleophilic attack at C-17 position of pregnenolone (Fig. 3). 5-Androsten-3β,17α-diol is than the real precursor of epitestosterone.

6. The metabolism of epitestosterone

Little is known about the metabolism of epitestosterone, especially in the human. In human term placenta epitestosterone could be aromatized to estradiol-17α [33]. In normal men injected with labelled epitestosterone approximately 50% of the injected radioactivity was recovered as unchanged epitestosterone, whereas etiocholanolone plus androsterone represented only 2%, while 5β- and 5α-androstane-3α,17α-diols accounted for about 5%. Epitestosterone is poorly metabolized in man and it is not metabolised in measurable amounts to either 16-androsten-3α-ol or testosterone [73].

In in vitro experiments with rat liver slices and liver subcellular fractions, the reduction of the 3-oxogroup and 4-ene double bond was observed, though to a lesser extent than in testosterone, and no formation of 17 oxo-compounds could be demonstrated [60–62]. No formation of 16-ene steroids could be documented in the testes of bulls, rabbits or rats [61]. In none of the experiments in rats with radioactive epitestosterone and testosterone a conversion of 17α-hydroxy group to the corresponding 17β-hydroxy group or conversely was found [60–62]. Analogously, mouse liver was inactive in the interconversion of epitestosterone and testosterone, whereas in mouse kidney epitestosterone was the major metabolite of testosterone and trace quantities of 5α-androstane-3α,17α-diol and 5α-androstane-3β,17α-diol were indicated, but not conclusively identified.

7. Biological activity of epitestosterone

It has been believed that epitestosterone is virtually devoid of any biological activity; especially as no androgenic action could be demonstrated [23]. A marginal note existed indicating that epitestosterone is an inhibitor of 5α-reductase [49]. In 1965 Kincl et al. [41] injected epitestosterone, as well as several other androgens, estrogens and anabolic steroids, into 5 days old male and female rats and evaluated the steroid in vivo action on gonads at the age of 45 days. Epitestosterone was active at rather high dose of 5 mg and its activity was more distinctly expressed in the females than in males. In spite of the fact that epitestosterone of all tested androgens and anabolics most effectively inhibited the luteinization, decreased the weight of ovaries and increased body weight and weight of uterus, the findings were neither commented or further investigated more in detail. Further unspecified antigonadotropic activity of epitestosterone was postulated by the US patent as early as in 1959 [1].

7.1. Antiandrogenic activity

In 1987 two dermatologists [51] observed that subcutaneously implanted epitestosterone prevents an androgen stimulation of the flank organ of the golden Syrian hamster as expressed by pigmentation, sebaceous gland growth and hair follicle diameter. As epitestosterone not only blocked the action of testosterone, but also antagonized the action of dihydrotestosterone, the effect could not be accounted for by known 5α-reductase inhibition. For this reason the question of antiandrogenic activity of epitestosterone was re-examined [4,5,58,59,64]. In classical design of antiandrogenic activity testing epitestosterone reduced the effect of testosterone propionate on body weight increments of castrated male mice. The relative organ weights indicated significant antiandrogenic and antirenotropic activities of epitestosterone [63]. The relative weights of seminal vesicles and kidneys were significantly reduced in comparison with the group of castrated mice receiving testosterone propionate alone. The effect was more pronounced than that of the antiandrogenic action of cyproterone acetate [58,59,64]. The relative binding affinity of epitestosterone to rat prostate cytosol receptor using ^{3}H methytrienolone as a ligand revealed K_i 29.8 nmol/l and relative binding slightly higher than that of cyproterone acetate. In vitro experiments with 5α-reductase from rat prostate [58] confirmed the earlier observations [49] that epitestosterone is an effective competitive inhibitor of testosterone conversion to dihydrotestosterone. Using corticosterone as a substrate, epitestosterone displayed a weak inhibitory activity on 11β-hydroxysteroid dehydrogenase with K_i 850, 1200 nmol/l and V_{max} 2420, 3900 nmol/l min for renal and testicular enzyme, respectively [3]. In experiments with male mice, epitestosterone administration reduced the secretion of LH and FSH [64]. In females the antigonadotropic activity showed an interesting biphasic effect, more distinctly on LH than FSH secretion in estrogen primed rats [5], perhaps by different dose-dependent action on hypothalamus and hypophysis levels. Epitestosterone inhibited also 17α-hydroxylation and C-19–20-desmolase in rat testes subcellular fractions [4]. This effect has also been demonstrated also in human testicular tissue. Epitestosterone injected to pregnant rats caused a decrease of androgen dependent organ weights (seminal vesicles, prostate) and caused small morphological disturbances in epididymis development. In mice, epitestosterone decreased the bone density, ash weight, and calcium and phosphate content of femoral bone tissue significantly, although not to values as low as those seen in castrated animals [12].

A significant negative correlation between epitestosterone and estradiol levels in human male serum has been found [34], but no inhibition of aromatase was observed [6]. In establishing a theory to predict male-pattern baldness, Choi et al. [14] investigated the correlation of testosterone, epitestosterone, and dihydrotestosterone with 5α-reductase in hair using gas chromatography/mass spectrometry. Hair samples were obtained from a group of balding subjects and their sons, as well as from a corresponding age-matched, non-balding group. The ratio of testosterone to epitestosterone was significantly greater (mean 46.41, $P < 0.001$; mean 35.83, $P < 0.001$, respectively) in the hair of balding fathers and their sons than in the hair of the non-balding control subjects (mean 9.17 and 10.47, respectively). These findings demonstrate that analysis of terminal hair may not only provide a basis for predicting baldness when the subject is still young, but also for preventing and treating male-pattern baldness by controlling the steroid hormone balance.

Hammond et al. [31] investigated whether male hormones could be neuroprotective analogously to estrogens and tested the effects of testosterone, methyltestosterone and epitestosterone at physiological concentrations on primary cultures of human neurons induced to undergo apoptosis by serum deprivation. In contrast to flutamide, which eliminated testosterone-mediated neuroprotection, epitestosterone showed a slight neuroprotective effect but not through the androgen receptor.

Besides epitestosterone activity exerted by androgen receptor mechanism, a non-genomic mechanism has been described for epitestosterone uterine relaxation action [54].

These pluripotent mechanisms by which androgen counteracting activity of epitestosterone is exerted indicate that epitestosterone may be classified as a hormone which can contribute to the regulation of androgenic action, e.g. in biological processes in ageing prostates or in differentiation of hair follicle sensitivity to androgen action.

Acknowledgements

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