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Effects of androstenedione administration on epitestosterone metabolism in men

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Abstract

Androstenedione is a steroid hormone sold over-the-counter to individuals who expect that it will enhance strength and athletic performance. Endogenous androstenedione is the immediate precursor of testosterone. To evaluate the metabolism of oral androstenedione, we randomly assigned 37 healthy men to receive 0 (group 1), 100 mg (group 2), or 300 mg (group 3) of androstenedione in a single daily dose for 7 days. Eight-hour urines were collected 1 day before the start of androstenedione, and on days 1 and 7. Using gas chromatography–mass spectrometry, we measured excretion rates of glucuronide-conjugated epitestosterone, its putative precursor (E-precursor), and metabolites (EM-1 and EM-2), and we evaluated possible markers of androstenedione administration. Day 1 and 7 rates were not different: the means were averaged. The means (μg/h) for groups 1, 2, and 3, respectively were, for epitestosterone 2.27, 7.74, and 18.0; for E-precursor, 2.9, 2.0, and 1.5; for EM-1/E-precursor 0.31, 1.25, and 2.88; for EM-2/E-precursor 0.14, 0.15, and 1.15; for testosterone/epitestosterone (T/E) 1.1, 3.5, and 3.2. Epitestosterone, EM-1, and EM-2 excretion was greater in groups 2 and 3 versus group 1 ($0.0001 < P < 0.03$), as were EM-1/E-precursor, EM-2/E-precursor, and T/E. E-precursor excretion was lower in groups 2 ($P = 0.08$) and 3 ($P = 0.047$) versus group 1. Androstenedione increases excretion of epitestosterone and its two metabolites, while decreasing that of its precursor. Elevated ratios of EM-1- and EM-2/E-precursor, and the presence of 6α-hydroxyandrostenedione are androstenedione administration markers.

Keywords: Androstenedione; Epitestosterone; Steroids; Metabolism; Doping; Testosterone/epitestosterone ratio

1. Introduction

Androstenedione, a steroid hormone, is an immediate precursor to testosterone in the intrinsic synthetic pathways of androgens [1,2]. In the United States, androstenedione is sold over-the-counter and the sport supplement market attained annual sales of 1.4 billion in 1999 [3]. The media suggests that sales continue to grow at a prodigious rate. Androstenedione is marketed primarily to athletes as a potential anabolic agent. Advertising materials claim that androstenedione improves athletic performance, libido, and quality of life; however, none of these assertions have been demonstrated in peer-reviewed studies.

Many sport organizations prohibit the use of androstenedione and some test the urine of athletes for steroids, however the prohibition is difficult to enforce because consensus on the criteria for a positive case of androstenedione use has not been attained. Potential urinary markers of androstenedione administration include extremely high levels of testosterone, DHT, androsterone, and etiocholanolone [4]. In some individuals, androstenedione use increases the ratio of testosterone to epitestosterone (T/E) above the International Olympic Committee (IOC) cut-off of 6 [5,6], as often occurs in subjects who use testosterone [7]. In those men, the T/E ratio increases because of both an increase in urinary testosterone excretion and a decrease in urinary epitestosterone excretion. Epitestosterone is of great interest in the field of doping control because it is the denominator in T/E, a surrogate marker of testosterone administration. Yet, while there is much information on urinary testosterone, relatively little is known about epitestosterone.

Although epitestosterone was first isolated from urine in

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1964 [8], its physiological role has not been established. It has minimal androgenic properties in man [9] and it is produced in both the adrenals and ovaries [8]. The administration of ACTH, hCG, or an i.v. infusion of epitestosterone increases urinary epitestosterone [10]. The production rate of epitestosterone has been estimated at just 200–300 μg/day [10], or 5% of the production rate of testosterone. No precursors of epitestosterone have been conclusively identified, but 5-androsten-3β,17α-diol (E-precursor) has been proposed as a possible precursor [11]. It has also been suggested that epitestosterone might arise as a byproduct of 16-androstene synthesis [12]. In animals**,** epitestosterone inhibits 5α-reductase and has some anti-androgen activity [13].

We recently demonstrated that oral androstenedione administration increases serum testosterone and testosterone glucuronide levels, and markedly increases the urinary excretion rates of the glucuronides of testosterone, DHT, androsterone, and etiocholanolone [4,14]. In the same subjects we showed that trace contamination (0.001%) of androstenedione with 19-norandrostenedione is sufficient to cause urine test results positive for 19-norandrosterone, the standard marker for nandrolone use [15]. In this study we examine the effect of oral androstenedione administration on the urinary excretion of glucuronides of epitestosterone, epitestosterone metabolites, and E-precursor. In addition, we examine the effect of oral androstenedione administration on the T/E ratio and discuss diagnostic criteria for the detection of androstenedione administration.

2. Experimental

2.1. Androstenedione study subjects

The details of the subjects and protocol have been reported previously [14] and are summarized below. The original study enrolled 42 men (37 Caucasians, 2 African Americans, and 3 Asians) between 20 and 40 years of age recruited through postings at Massachusetts General Hospital and affiliated institutions, and advertisements in local newspapers. Of these, 37 completed all urine collections and are included in the present report. All subjects denied participation in competitive weightlifting or bodybuilding. Men with a history in the previous 6 months of cardiopulmonary disease, malignancy, prostate disease, major psychiatric disease, substance abuse, or use of any medication known to affect steroid hormone or binding protein levels were excluded. Subjects were also excluded if they reported prior use of androstenedione or androgenic anabolic steroids. All subjects were required to have normal serum testosterone and creatinine levels, and normal liver function tests. The study was approved by the Human Research Committee at Massachusetts General Hospital, and all study participants gave written informed consent. In addition 68 urines from healthy male medical students participating in a biochemistry course were analyzed. These samples were collected under the oversight of the UCLA Human Subjects Protection Committee.

2.2. Study protocol

Subjects were randomly assigned to one of three groups: no androstenedione (group 1, $n = 13$), 100 mg of androstenedione (Sports One®, 99.9% purity) daily for 7 days (group 2, $n = 13$), or 300 mg of androstenedione daily for 7 days (group 3, $n = 11$). On each of the seven treatment days, the androstenedione capsules were dispensed by a nurse at the same time of day (within a 2-h window). Subjects were instructed to take nothing by mouth except water for 1 h after androstenedione administration. Urine samples were collected for 8 h on the day prior to the first treatment and on days 1 and 7 during androstenedione administration. The urinary excretion rates of the glucuronides of epitestosterone, E-precursor, and epitestosterone metabolites, namely 5β-androstan-3α,17α-diol (EM-1) and 5α-androstan-3α,17α-diol (EM-2) were measured.

2.3. Identification of urinary steroids

Epitestosterone precursor, EM-1, EM-2, and 6-hydroxyandrostenedione were identified in urine extracts as their per-TMS derivatives through gas chromatography–mass spectrometry (GC–MS, Finnigan MAT 95) by comparison of spectra and retention times of compounds in subject urines to those of reference standards. In addition, 6α- and 6β-hydroxyandrostenedione were distinguished from one another as their mono-TMS derivatives.

2.4. Quantitative analysis of urinary steroids

Urinary steroid concentrations varied widely, therefore some analyses were repeated after dilution with steroid-free urine. Forty ng/ml of internal standard (16,16,17-$^2H_3$-testosterone) were added to each 2.5 ml of urine or diluted urine and steroids were extracted using a modification of a previously reported method [16]. The procedure includes C-18 (Varian Associates) column chromatography, elution with methanol, enzymatic hydrolysis with β-glucuronidase from *E. coli*, extraction with diethyl ether, solvent evaporation, and desiccation in vacuo. Next, the per-TMS derivatives were prepared. Two μl from 50 μl total were injected with a 20:1 split at 280°C on a HP5890 Series II gas chromatograph equipped with a 17-m HP-Ultra 1 column coupled to a Finnigan MAT 95 mass spectrometer operating at 3000 resolution in multiple ion detection mode. We monitored m/z 432.3, 256.2, 256.2, and 254.2 for epitestosterone, EM-1, EM-2, and EP, respectively, and m/z 518.3, 503.3, and 319.2 for 6-hydroxyandrostenedione. Because the urine samples were analyzed after hydrolysis, free and glucuronide-conjugated urinary steroids were initially measured as their sum. The T/E ratio was determined from the

peak height ratio of testosterone (m/z = 432.3)/epitestosterone (m/z = 432.3). Urinary concentrations were determined using a standard curve. The method limits of detection for testosterone, epitestosterone, E-precursor, EM-1, EM-2 and 6α-hydroxyandrostenedione were 0.3, 0.3, 0.8, 0.4, 0.4 and 2.2 ng/ml, respectively. The method CV was 3% for testosterone and ranged between 8 and 10% for all other compounds. Recoveries were 85%, 74% and 70% for E-precursor, EM-1 and EM-2, respectively and $>$ 90% for testosterone, epitestosterone and 6α-hydroxyandrostenedione. Unconjugated urinary free testosterone and epitestosterone were quantified from a separate extract for 3 subjects in each group.

2.5. Statistical analysis

There was no significant difference in urinary excretion rates for any of the steroids between the means of days 1 and 7, therefore rates from days 1 and 7 were averaged. There was no significant difference between the day 1 and day 7 means for T/E, EM-1/E-precursor, or EM-2/E-precursor, therefore days 1 and 7 were averaged. Excretion rates of urinary steroids were compared using a repeated measures analysis of covariance with the baseline value as the covariate. Data are expressed as the mean ± SEM. The P values are for the difference between the means of the three groups (1 vs. 2, 1 vs. 3, and 2 vs. 3). All P values are 2-sided and values $<$0.05 are considered statistically significant.

3. Results

The baseline characteristics of the study subjects have been reported previously. All subjects were between the ages of 20 and 40 and well matched for baseline serum testosterone, androstenedione, estrone, and estradiol concentrations [14].

3.1. Epitestosterone, its metabolites, and precursor

Urinary excretion rates of epitestosterone, EM-1, and EM-2, were greater in subjects who received androstenedione than in the controls ($P < 0.02$ for all comparisons between groups 1 and 2, and $P < 0.003$ for all comparisons between groups 1 and 3; Fig. 1). Excretion rates of epitestosterone increased 3-fold and 8-fold in groups 2 and 3, respectively. In group 3, the mean baseline urinary excretion rate of epitestosterone was 2.6 ± 0.4 μg/h and it increased to 18 ± 2.1 μg/h after androstenedione administration. The excretion rate of 5-androsten-3β,17α-diol was significantly lower in group 3 than group 1 ($P < 0.05$), but not in group 2 ($P = 0.08$). Less than 0.1% of the measured urinary epitestosterone was present in the form of free epitestosterone in subjects receiving either dose of androstenedione. Excretion rates of free epitestosterone did not differ among groups. The excretion rate of EM-1 increased

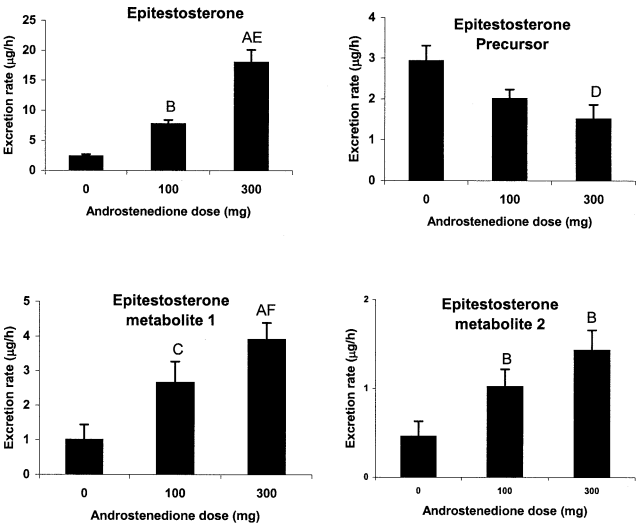

Fig. 1. Mean ± SEM of urinary excretion rates for epitestosterone, epitestosterone metabolite 1, epitestosterone metabolite 2, and epitestosterone precursor. [A] $P < 0.001$ compared to controls; [B] $P < 0.003$ compared to controls; [C] $P < 0.02$ compared to controls; [D] $P < 0.05$ compared to controls; [E] $P < 0.0001$ compared to 100-mg dose group; [F] $P < 0.05$ compared to 100-mg dose group.

3-fold in group 2 and 4-fold in group 3 (Fig. 1). The excretion rate of EM-2 also increased significantly.

3.2. Ratios

Both EM-1/E-precursor and EM-2/E-precursor increased with the dose of androstenedione (Fig. 2). EM-1/E-precursor was greater in group 2 ($P < 0.01$) and group 3 ($P < 0.0001$) than in group 1. Similarly, EM-2/E-precursor was greater in group 2 ($P < 0.02$) and group 3 ($P < 0.0001$) than in group 1. In addition, both EM-1/E-precursor ($P < 0.0001$) and EM-2/E-precursor ($P < 0.002$) were greater in group 3 than in group 2. The mean EM-1/E-precursor and EM-2/E-precursor in the baseline urines was 0.3 (SD = 0.4) and 0.2 (SD = 0.2), respectively. The mean EM-1/E-precursor and EM-2/E-precursor ratios were at least 4 SD above the mean in 20/48 and 13/48 post androstenedione administration urines, respectively.

The T/E ratio was greater in group 2 ($P < 0.0001$) and group 3 ($P < 0.001$; Fig. 2) than in group 1, though there was no significant difference in T/E between groups 2 and 3. The T/E ratio increased in 22 of the 24 subjects who received androstenedione and exceeded 6 in 1 of 13 subjects in group 1 and 3 of 11 subjects in group 3. The two subjects in whom T/E did not increase were Asian (group 3, Fig. 3). Unlike all other subjects in group 3, their T/E declined after androstenedione administration. The mean T/E for groups 2 and 3 was 3.5 ± 0.4 and 3.2 ± 0.7 (3.9 ± 0.7 without the two Asians), respectively. The baseline excretion rates of epitestosterone, EM-1, EM-2, and E-precursor in the 2 Asian men were lower than all but one other subject in group 3. After androstenedione administration, the percent

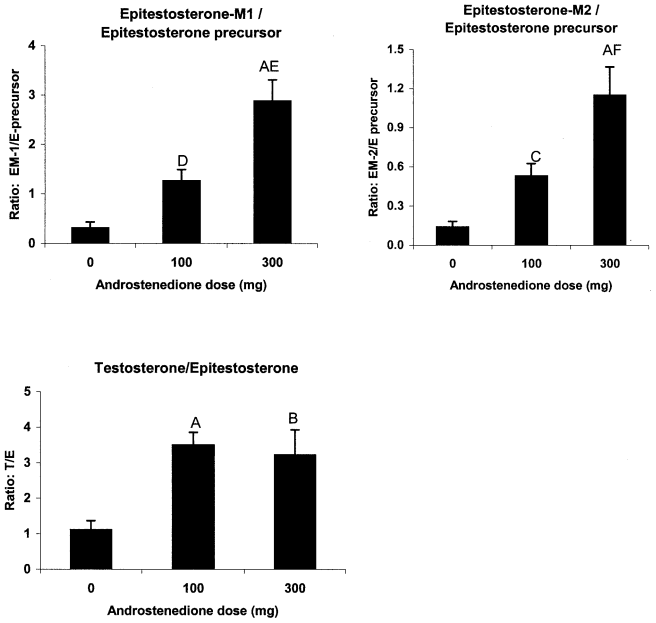

Fig. 2. Mean ± SEM of urinary ratios: EM-1/E-precursor, EM-2/E-precursor, and T/E. [A] $P < 0.0001$ compared to controls; [B] $P < 0.001$ compared to controls; [C] $P < 0.02$ compared to controls; [D] $P < 0.01$ compared to controls; [E] $P < 0.0001$ compared to 100-mg dose group; [F] $P < 0.002$ compared to 100-mg dose group.

increases in their epitestosterone (or decrease in the case of E-precursor), EM-1/E-precursor, and EM-2/E-precursor were similar to those of other group 3 subjects.

3.3. Identification of 6α-hydroxyandrostenedione

6α-Hydroxyandrostenedione was identified in the urine of four subjects in group 3 by its retention time and mass spectrum and subsequently identified by selected ion monitoring (SIM) of three characteristic ions in the urine of all 24 subjects who received androstenedione. It was not found in any of the urines collected from the control subjects, in the baseline urines from the subjects who received androstenedione, or in any of the 68 medical student urines. 6β-Hydroxyandrostenedione was not found in any of the urines studied.

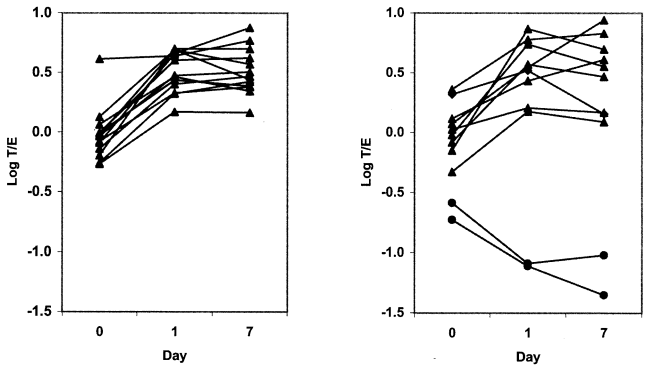

Fig. 3. Log urinary T/E ratios on the baseline day and on day 1 and 7 of administration of either 100 mg (left panel) or 300 mg (right panel) of androstenedione. The solid circles in the right panel represent Asian subjects.

4. Discussion

4.1. Epitestosterone

This study demonstrates that both 100-mg and 300-mg doses of androstenedione increase the urinary excretion rate of epitestosterone and decrease the excretion rate of its putative precursor, E-precursor. Approximately 20% of the epitestosterone produced daily is recovered in urine as its glucuronide [10]. The 8-fold increase in the epitestosterone excretion rate in group 3 corresponds to an epitestosterone production rate of 1500 μg/day. The increased production most likely arises from direct metabolism of androstenedione to epitestosterone although it is not clear which enzymes are responsible for this conversion.

One enzyme that is a likely candidate to convert androstenedione to epitestosterone is 3(17)α-hydroxysteroid dehydrogenase (HSD). This enzyme is found in liver and kidney tissue from rabbits and hamsters [17–19]. Although this HSD exhibits both 3α- and 17α-HSD activities, only epitestosterone is formed when the substrate is androstenedione [17]. Another possible candidate is a 17α-HSD, an enzyme that was recently isolated and fully characterized from an intestinal *Eubacterium* sp. [20]. Although the tissue distribution of these enzymes has not been fully described in humans, the ovary and blood are known sites of androstenedione to epitestosterone conversion [21,22]. It is very unlikely that androstenedione is converted to epitestosterone via testosterone because several attempts to demonstrate this pathway have failed [5,10,23]. Nevertheless, other potential pathways are possible.

4.2. E-precursor

E-precursor is secreted by the testes [11,24] and, although its exact origin is unclear, it is an obligatory side-product of the synthesis of 5,16-androstadien-3β-ol (ADL) from pregnenolone [12]. Further, correlated reduction in 5-androsten-3β,17α-diol and epitestosterone excretion (after testosterone administration) [7] is consistent with the hypothesis that 5-androsten-3β,17α-diol is a precursor of epitestosterone [11]. If concomitant production of ADL and E-precursor as proposed by Weuston et al. [12] is the only physiological source of E-precursor, then we would expect ADL production to decrease along with the observed decrease in E-precursor production. However, the increase in epitestosterone excretion as excretion of E-precursor declined indicates that epitestosterone is arising from another source, most likely exogenous androstenedione.

4.3. Putative epitestosterone metabolites

If the metabolism of epitestosterone follows pathways parallel to those of testosterone metabolism, with the only difference being that epitestosterone is the 17α-epimer of testosterone, then we would expect 5β and 5α reduction of epitestosterone to produce EM-1 and EM-2, respectively. The fact that androstenedione administration increases the excretion rates of EM-1 and EM-2 in addition to increasing that of epitestosterone supports this hypothesis. EM-1 and EM-2 are not likely to be the metabolites of one of the other steroids whose excretion rate increases with androstenedione administration, e.g. androsterone, because androsterone has been shown to be an end-metabolite [25]. Epitestosterone administration increases urinary 17α-diols, although they could not be unambiguously identified by the available methods [10]. EM-1 and EM-2 are found in urine by GC–MS after testosterone administration [7], although their excretion rates do not decrease in parallel with that of epitestosterone.

4.4. Ratio of testosterone to epitestosterone

In sport drug testing, a T/E ratio greater than 6 is accepted as evidence that an athlete has used exogenous testosterone. In this study, the T/E ratio increased in 22 of the 24 subjects who received androstenedione though only 4 of 24 men had a ratio greater than 6. In a recent study, T/E ratios of 6 and 22 were observed in two subjects who received 50 mg of androstenedione orally [26]. Thus the T/E ratio is a non-specific marker of testosterone, androstenedione, DHEA, and androstenediol administration [26–30]. Athletes should be aware that over-the-counter steroids such as androstenedione might increase T/E ratios above the cut-off of 6.

Previously we reported that urinary testosterone excretion was lower in two Asian subjects compared with nine non-Asians [4], and that it increased much less in the Asians than in the non-Asians after androstenedione administration. In this study, the T/E ratio declined in the same two Asians because their epitestosterone levels rose higher and faster than testosterone. The reason for this apparent ethnic difference in androstenedione metabolism is not known.

4.5. Strategy for detecting androstenedione administration

The EM-1/E-precursor and EM-2/E-precursor ratios are potential markers of androstenedione administration, however, if a very conservative cut-off of +4SD above the pre-treatment mean were used, only 27–42% of the post-androstenedione administration urines in our study exceed the cut-off. Administration of epitestosterone, which is prohibited by most sport organizations, might also increase the EM-1/E-precursor and EM-2/E-precursor ratios.

6α-Hydroxyandrostenedione appears to be a specific marker for androstenedione administration. It was not detected at a limit of detection of 2.2 ng/ml in any of the baseline urine samples from the 37 subjects and it was present in the urines of all subjects who received either dose of androstenedione. Moreover, 6α-hydroxyandrostenedione was not found in the urines from an additional 68 control males, a finding that provides strong evidence that it is not an endogenous urinary steroid. These findings suggest that 6α-hydroxyandrostenedione may be a sensitive and specific marker of exogenous androstenedione use that could be used to test athletes.

Other investigators have reported a 10–60 fold increase in androstenedione excretion rate after androstenedione administration [27,28]. When we used SIM, as they did, we found a steroid with a retention time and SIM scan similar to those of androstenedione; but using the more informative full scan mode, it turned out not to be androstenedione. Other investigators have reported the presence of hydroxylated metabolites of androstenedione [31], including 6α-hydroxyandrostenedione [32], after androstenedione administration. We found one other compound in the post-androstenedione urine samples. Its mass spectrum was consistent with that of a steroid, but it did not match any of the steroid spectra in our library. Two other steroids yet to be identified were either absent in baseline urine and present after androstenedione administration, or present in baseline urine but greatly increased after androstenedione administration.

The administration of androstenedione to healthy male subjects increases the urinary excretion rates of epitestosterone and two putative metabolites of epitestosterone, and decreases the excretion rate of a putative precursor of epitestosterone. In addition the T/E ratio and the ratio of the putative metabolites to the precursor increase. The increases in the epitestosterone metabolites to precursor ratios may be useful as markers of androstenedione administration but they lack sensitivity. 6α-Hydroxyandrostenedione was identified in all post-androstenedione urines, but was not detected in any of the control urines, thus it appears to be a specific marker of androstenedione administration.

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