0021-972X/05/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 90(2):855–863

doi: 10.1210/jc.2004-1577

Δ-4-Androstene-3,17-Dione Binds Androgen Receptor, Promotes Myogenesis *in Vitro*, and Increases Serum Testosterone Levels, Fat-Free Mass, and Muscle Strength in Hypogonadal Men

Ravi Jasuja,* Pandurangan Ramaraj,* Ricky Phong Mac,* Atam B. Singh,* Thomas W. Storer,* Jorge Artaza, Aria Miller, Rajan Singh, Wayne E. Taylor, Martin L. Lee, Tina Davidson, Indrani Sinha-Hikim, Nestor Gonzalez-Cadavid, and Shalender Bhasin

Division of Endocrinology, Metabolism, and Molecular Medicine (R.J., P.R., R.P.M., A.B.S., T.W.S., J.A., A.M., R.S., W.E.T., M.L.L., T.D., I.S.-H., N.G.-C., S.B.), Charles R. Drew University of Medicine and Science, Los Angeles, California 90059; and Laboratory for Exercise Science (T.W.S.), El Camino College, Torrance, California 90502

Previous studies of Δ4-androstene-3,17-dione (4-androstenedione) administration in men have not demonstrated sustained increments in testosterone levels, fat-free mass (FFM), and muscle strength, and failure to demonstrate androstenedione's androgenic/anabolic effects has stifled efforts to regulate its sales. To determine whether 4-androstenedione has androgenic/anabolic properties, we evaluated its association with androgen receptor (AR) and its effects on myogenesis *in vitro*. Additionally, we studied the effects of a high dose of 4-androstenedione on testosterone levels, FFM, and muscle strength in hypogonadal men.

We determined the dissociation constant (K_d) for 4-androstenedione using fluorescence anisotropy measurement of competitive displacement of fluorescent androgen from AR ligand-binding domain. AR nuclear translocation and myogenic activity of androstenedione were evaluated in mesenchymal, pluripotent C3H10T1/2 cells, in which androgens stimulate myogenesis through an AR pathway. We determined effects of a high dose of androstenedione (500 mg thrice daily) given for 12 wk on FFM, muscle strength, and hormone levels in nine healthy, hypogonadal men.

4-Androstenedione competitively displaced fluorescent androgen from AR ligand-binding domain with a lower affinity than dihydrotestosterone (K_d, 648 ± 21 and 10 ± 0.4 nM, respectively). In C3H10T1/2 cells, 4-androstenedione caused nuclear translocation of AR and stimulated myogenesis, as indicated by a dose-dependent increase in myosin heavy chain II+ myotube area and up-regulation of MyoD protein. Stimulatory effects of 4-androstenedione on myosin heavy chain II+ myotubes and myogenic determination factor expression were attenuated by bicalutamide, an AR antagonist. Administration of 1500 mg 4-androstenedione daily to hypogonadal men significantly increased serum androstenedione, total and free testosterone, estradiol, and estrone levels and suppressed SHBG and high-density lipoprotein cholesterol levels. 4-Androstenedione administration was associated with significant gains in FFM (+1.7 ± 0.5 kg; $P = 0.012$) and muscle strength in bench press (+4.3 ± 3.1 kg; $P = 0.006$) and leg press exercises (+18.8 ± 17.3 kg; $P = 0.045$).

4-Androstenedione is an androgen that binds AR, induces AR nuclear translocation, and promotes myogenesis *in vitro*, with substantially lower potency than dihydrotestosterone. 4-Androstenedione administration in high doses to hypogonadal men increases testosterone levels, FFM, and muscle strength, although at the dose tested, the anabolic effects in hypogonadal men are likely because of its conversion to testosterone. (*J Clin Endocrinol Metab* 90: 855–863, 2005)

UNTIL RECENTLY, Δ4-androstene-3,17-dione (4-androstenedione), a precursor of testosterone in the androgen biosynthetic pathway, was sold as a dietary supplement under the Dietary Supplement Health and Education Act (1, 2), although the Food and Drug Administration has recently restricted its over-the-counter sales. Androstenedione's purported performance-enhancing effects and its easy availability in nutrition stores and over the internet made it a popular substance among athletes and recreational bodybuilders (3, 4). Unlike other androgenic steroids, whose sales are regulated within the dictates of the Controlled Substances Act, 4-androstenedione sales had not been subject to the regulatory oversight of the Food and Drug Administration and the Drug Enforcement Administration. 4-Androstenedione meets some of the criteria of the definition of anabolic steroid as set forth in the Controlled Substances Act (5); it has chemical and pharmacological similarity to testosterone and it is not an estrogen, a progestin, or a corticosteroid, but we do not know whether it meets the fourth essential criterion for classification as an anabolic steroid, *i.e.* promotion of muscle growth. Lack of verifiable evidence of its anabolic/androgenic potency has stifled efforts at regulatory control of its sales. The ability of 4-androstenedione, either alone or in conjunction with resistance exercise training, to increase

First Published Online November 2, 2004

* R.J., P.R., R.P.M., A.B.S., and T.W.S. contributed equally to this work.

Abbreviations: AR, Androgen receptor; $C_{average}$, average concentration; DEXA, dual-energy x-ray absorptiometry; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; FA, Fluormone AL green; FFM, fat-free mass; HDL, high-density lipoprotein; hpf, high-power field; LBD, ligand-binding domain; LBM, lean body mass; LDL, low-density lipoprotein; MHC, myosin heavy chain II; MyoD, myogenic determination factor; 1-RM, one-repetition maximum.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

muscle mass and strength and muscle protein synthesis has not been demonstrated (6–8). 4-Androstenedione is converted endogenously to testosterone, a potent androgen known to increase muscle size and strength. Previous studies of 4-androstenedione administration in eugonadal men have reported either small or no increments in testosterone concentrations (7, 9–16); however, these studies used relatively small doses (100–300 mg) of 4-androstenedione, whereas athletes use daily as much as 1500 mg androstenedione or more (4).

Because of its structural similarity to testosterone, and its virilizing effects in females of several mammalian species (17–19), we hypothesized that 4-androstenedione is an androgen and evaluated whether it possesses the essential properties of an androgen. First, we tested the ability of androstenedione to bind ligand-binding domain (LBD) of androgen receptor (AR). Second, we compared its myogenic potency in an *in vitro* bioassay using a mesenchymal pluripotent cell line, in which testosterone and dihydrotestosterone (DHT) stimulate formation of myotubes and expression of myogenic markers (20). Finally, we evaluated in healthy, hypogonadal men effects of a higher dose of 4-androstenedione (500 mg thrice daily) than had been used in previous studies, on fat-free mass (FFM) and muscle strength. Because 4-androstenedione administration to eugonadal men suppresses LH secretion and endogenous testosterone production, making it difficult to detect small increments in testosterone levels, we performed these studies in hypogonadal men with the assumption that increments in testosterone concentrations would be more easily demonstrable in hypogonadal men.

Subjects and Methods

Androstenedione purity, formulation, and dispensing

4-Androstenedione was purchased as bulk powder from Kronos Research Pharmacy (Las Vegas, NV). The analytical HPLC report provided by the manufacturer indicated that the powder contained more than 99.6% 4-androstenedione by weight. We further analyzed the powder by electron ionization mass spectrometry using a MicroMass Autospec mass spectrometer under the following conditions: electron energy, 70 eV; source temperature, 170 C; magnetic scanning conducted to span a mass range of 50–350 m/z; and cycle time, 1.5 sec. This analysis revealed no detectable amounts of testosterone, DHT, androstenediol, dehydroepiandrosterone (DHEA), or 3α-androstanediol.

For human studies, Kronos Research Pharmacy compounded 4-androstenedione as 500-mg capsules, using pharmaceutical-grade cellulose and gelatin as fillers.

AR binding in fluorescent polarization assay

Association constants for 4-androstenedione were determined using fluorescence anisotropy measurement of competitive displacement of fluorescent androgen analog [Fluormone AL green (FA); Invitrogen, San Diego, CA] from LBD of AR (PanVera Corp., Madison, WI). DHT and androstenedione were titrated into 25 nM AR-LBD solution containing 1 nM FA and 2 mM dithiothreitol. Polarization values of AR-LBD-bound FA were measured at graded ligand concentrations using Tecan Genios Pro (λ_{ex} = 485 nm; λ_{em} = 535 nm) to obtain IC_{50}, the concentration that results in a half-maximal shift in polarization value. Three competition curves from different batches of AR-LBD were constructed and fit to the following equation (21):

$$Y = mP_{100} + \frac{mP_0 - mP_{100}}{1 + 10^{(\ln(IC_{50} - C)H)}}$$

Y is measured in millipolarization units (mP); [C] is concentration of competitor; mP_0 is the highest limiting value of polarization (receptor:FA complex, ~98%) or 0% competitive displacement; mP_{100} is the lowest limiting value of polarization (receptor:FA complex, ~0%) or 100% competitive displacement. IC_{50} values obtained from the best fit for binding isotherm plots were used to evaluate dissociation constants (K_d) using the following equation:

$$K_d = \frac{K_{FA} IC_{50}}{K_{FA} + [L]}$$

where K_{FA} is K_d for FA and [L] is ligand concentration.

Effects of 4-androstenedione on myogenic and adipogenic differentiation of C3H10T1/2 pluripotent cells

C3H10T1/2 cells, grown in DMEM with 10% fetal bovine serum at 37 C, were treated with 20 μM 5-azacytidine for 3 d and seeded at 70% confluence in six-well plates or chamber slides (20). Cells were incubated with medium alone, androstenedione, or DHT without or with bicalutamide for 24 h for AR translocation studies, 3 d for assessment of myogenic determination factor (MyoD), and 14 d for assessment of myotubes and myosin heavy chain II (MHC) expression, as described previously (20).

For immunohistochemical analyses, cells grown in chamber slides were fixed in 2% paraformaldehyde, quenched with H_2O_2, blocked with horse or goat serum, and incubated with antibody for MHC (Vector Laboratories, Novo Castra, CA) or AR (Santa Cruz Biotechnology, Santa Cruz, CA) (20). Detection was based on secondary biotinylated antibody (1:200) followed by addition of streptavidin-horseradish peroxidase ABC complex (1:100) and 3,3-diaminobenzidine. Cells were counterstained with Mayer's hematoxylin. In negative controls, we either omitted the first antibody or used rabbit nonspecific IgG. Effects of androstenedione and DHT on myogenesis were assessed quantitatively by measuring area covered by MHC+ myotubes, using ImagePro (Media Cybernetics, Silver Spring, MD); the area of MHC+ myotubes was averaged over 10 fields. Adipocytes were stained with Oil Red O and counted in 10 fields.

For Western analysis, cell lysates (50–100 μg) were electrophoresed on 7.5% polyacrylamide gels (20). After transfer, membranes were incubated with 1:200 mouse monoclonal anti-MHC (fast MHC, Vector), 1:500 anti-AR (Santa Cruz Biotechnology), 1:500 anti-MyoD (Santa Cruz Biotechnology), or 1:10,000 anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula) antibody. Washed membranes were incubated with secondary antibody (1:1000) linked to horseradish peroxidase. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system.

Studies in hypogonadal men

All participants signed a consent form approved by the Institutional Review Board of Charles Drew University. This prospective, controlled trial took place between December 2000 and October 2003 in a setting of the Clinical Research Center and consisted of a screening phase, 2-wk control period, and 12-wk treatment period. The 12-wk treatment period was selected because in previous studies, this duration had been sufficient to observe testosterone effects on FFM (22, 23).

Treatment regimen

Subjects took one 500-mg capsule orally, thrice daily, the last dose at bedtime. The treatment duration was 12 wk. Subjects were given a 14-d supply of 4-androstenedione, which was renewed every 2 wk. Compliance was assessed by pill counts.

Participants

We recruited nine healthy, 19- to 65-yr-old, hypogonadal men with total testosterone level less than 300ng/dl. We excluded men with diabetes mellitus, prostate cancer, or American Urological Association symptom score greater than 7. Subjects receiving testosterone replacement were eligible if they discontinued androgen therapy for 12 wk. Subjects with other endocrinopathies were eligible if they had been on

stable hormone replacement regimens for at least 12 wk. We excluded men who were taking anabolic agents, including androgens, dietary supplements, or GH. Men who were participating in resistance exercise or moderate to intense endurance exercise were excluded.

Nutritional intake and exercise

Subjects were prescribed a diet standardized for energy (150 $kJ \cdot kg^{-1} \cdot d^{-1}$) and protein (1.3 $g \cdot kg^{-1} \cdot d^{-1}$). Dietary instructions were reinforced monthly. The men were asked not to undertake resistance training or moderate to heavy endurance exercise.

Outcomes

The principal outcome measures were changes in total and free testosterone levels, FFM, and maximal voluntary strength in leg press and chest press exercises. Serum androstenedione, total and free testosterone, estradiol, and estrone levels were measured at 0, 1, 2, 4, 6, and 8 h on d 0 and 84. Average concentration ($C_{average}$) of hormone on d 0 and 84 were calculated by computing area under the curve over the 8-h sampling period and dividing by 8. Body composition by dual-energy x-ray absorptiometry (DEXA) and muscle strength in bench press and leg press exercises were measured at baseline and on d 84. Physical examination, adverse events, plasma lipids, prostate-specific antigen (PSA), and blood counts and chemistries were evaluated at baseline and during wk 6 and 12.

Whole-body fat-free, lean, and fat mass were measured by DEXA (Hologic QDR4500A, Waltham, MA). Appendicular skeletal muscle and fat masses were determined manually by adding bilateral arm and leg lean and fat masses, respectively (24). The DEXA scanner was calibrated using a soft-tissue phantom before each scan.

We measured maximal voluntary strength in leg press and chest press exercises using one-repetition maximum (1-RM) (25) with pneumatic resistance (Keiser Sport, Fresno, CA). Because maximal voluntary strength measurements are highly effort dependent, several strategies were used to assure reliability and reproducibility and to minimize the confounding influence of the learning effect. Tests were performed in duplicate or triplicate, with careful attention to positioning so that starting knee flexion (90° by goniometry), the ensuing hip angles, and foot placement on the leg press footplate were standardized and held constant. The 1-RM procedure (25) included a familiarization period in which subjects were instructed in and then practiced the proper execution of the seated leg press exercise. After this familiarization, subjects completed a generalized warm-up consisting of 5 min of cycle ergometer or treadmill exercise plus stretching of the quadriceps, hamstrings, low back, and triceps surae. Immediately after this warm-up, subjects were positioned on the leg press machine with position measurements recorded for subsequent testing. The initial load was set at 50% of the subject's estimated 1-RM using reference values established in our laboratory. Subjects were first asked to perform eight repetitions of the leg press exercise at this load. After 1 min of rest, the subjects performed four repetitions at a load that was increased by approximately 20 kg. After a 2-min rest period, the load was increased again and attempts were then made to identify the 1-RM. Attempts were punctuated with 2-min rest intervals and continued until the 1-RM was identified as the greatest amount of weight lifted through the complete range of motion. Strength tests were conducted in duplicate on nonconsecutive days with scores required to be within 5%.

Biochemical analyses

Total testosterone was measured by a RIA (23) that has been validated against liquid chromatography-tandem mass spectrometry. Free testosterone, separated by equilibrium dialysis, was measured in dialysate by RIA (26). The cross-reactivities of 4-androstenedione, DHT, DHEA, and androstanediol in the testosterone assay were less than 0.1% for each. Cross-reactivities of testosterone, DHT, DHEA, and androstanediol were less than 0.1% in the androstenedione assay. SHBG and PSA levels

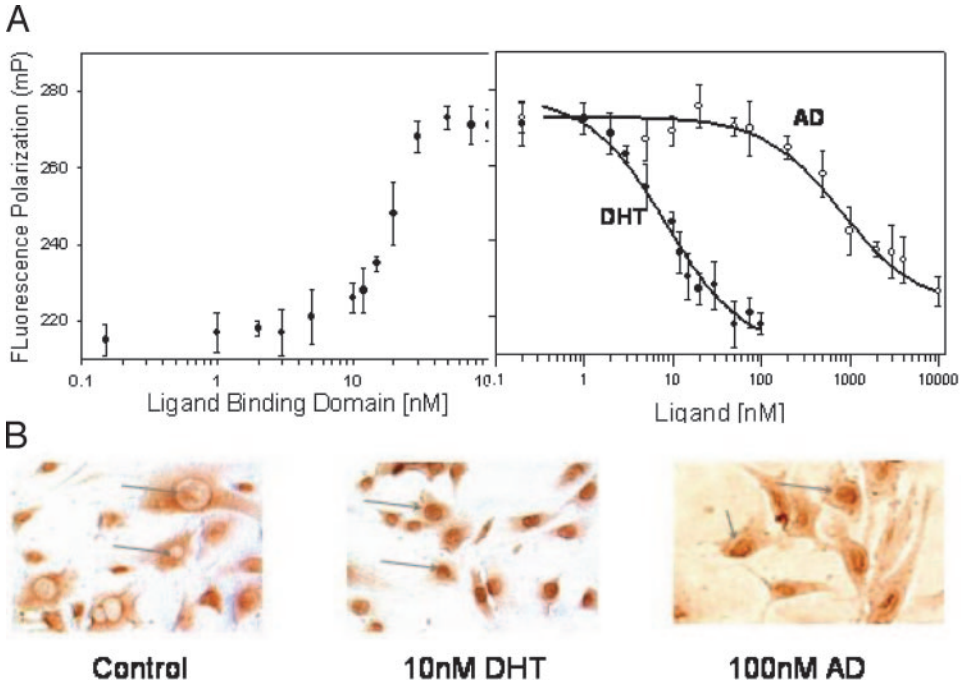

FIG. 1. A, Androstenedione binds AR with an affinity that is lower than that of DHT. Dissociation constants for androstenedione and DHT were calculated using fluorescence anisotropy measurement of competitive displacement of fluorescent androgen analog from AR-LBD. The *left panel* shows that the polarization value, recorded in millipolarization units (mP), is increased as increasing amounts of LBD are added to the FA solution. The *right panel* shows that DHT and androstenedione displace FA from LBD in a dose-dependent manner. K_d values for DHT and androstenedione were calculated as 10 ± 0.4 nM and 648 ± 21 nM. B, Androstenedione and DHT cause translocation of AR to the nucleus of C3H10T1/2 mesenchymal, pluripotent cells. C3H10T1/2 cells, grown in promyogenic conditions, were incubated for 24 h with medium alone, DHT (10 nM), or androstenedione (100 nM); fixed with 2% paraformaldehyde; and immunostained for AR, using anti-AR antibody. The *arrows* point to the nuclei; the nuclei in wells treated with medium alone (control) are not stained for AR; in contrast, in wells treated with DHT and androstenedione, the nuclei are immunopositive for AR, providing evidence of AR nuclear translocation. Magnification, ×200.

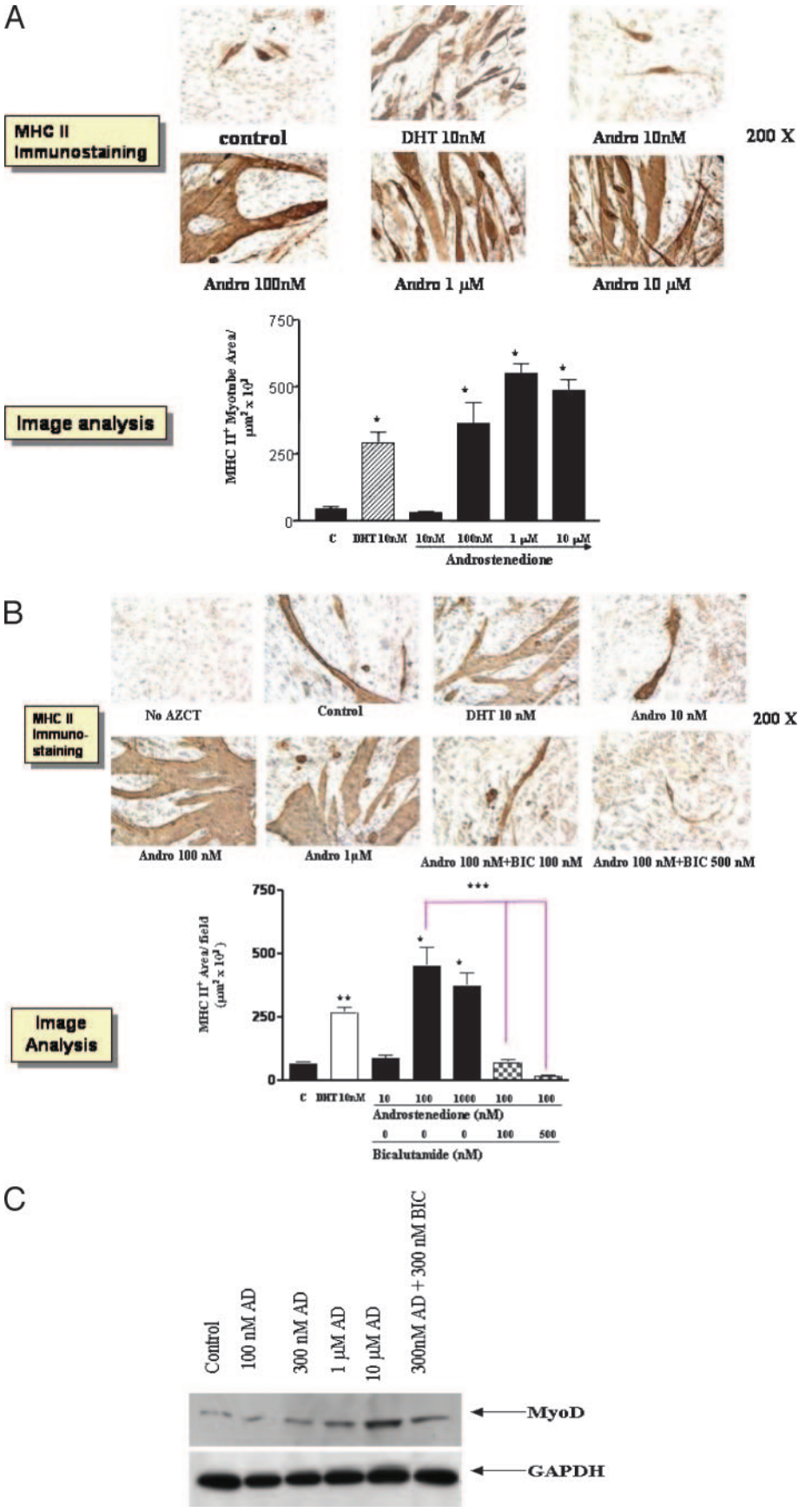

FIG. 2. Androstenedione promotes myogenesis in C3H10T1/2 mesenchymal, pluripotent cells, and its effects are blocked by bicalutamide. A, Androstenedione and DHT promote myogenic differentiation in C3H10T1/2 mesenchymal, pluripotent cells. The *upper* panel shows representative wells containing C3H10T1/2 cells, treated for 14 d with medium alone (control), 10 nM DHT, or graded concentrations of androstenedione (Andro), and analyzed for MHC expression by immunocytochemical staining, using an anti-MHC antibody. Magnification, ×200. The area covered by MHC+ myotubes was assessed by image analysis software (*lower panel*). Data are mean ± SEM (n = 6 for each concentration); *, P *vs.* control < 0.001. B, Androstenedione effects on myogenesis are blocked by bicalutamide. The *upper* panel shows representative wells containing C3H10T1/2 cells treated for 14 d with medium alone (control), DHT (10 nM), or graded concentrations of androstenedione (as shown) without or with bicalutamide (BIC) as shown. MHC expression was visualized by immunocytochemical staining (×200), using anti-MHC antibody. The *lower panel* shows the mean ± SEM area of MHC+ myotubes, measured by an image analysis software; *, $P < 0.001$ *vs.* control wells; **, $P < 0.01$ *vs.* control; ***, $P < 0.0001$ *vs.* wells treated with androstenedione plus bicalutamide. AZCT, 5′ azacytidine. C, Western blot analysis of MyoD protein. Cell extracts were analyzed by immunoblotting using anti-MyoD or anti-glyceraldehyde-3-phosphate dehydrogenase antibody. Androstenedione, DHT, or bicalutamide (BIC) concentrations (nM) are shown.

were measured by immunoradiometric assays. Sensitivities, and intra- and interassay coefficients of variation were as follows: total testosterone, 0.6 ng/dl (0.002 nmol/liter) and 8.2 and 13.2%; free testosterone, 0.22 pg/ml (0.8 pmol/liter) and 4.2 and 12.3%; 4-androstenedione, 0.05 ng/ml (0.2 nmol/liter) and 4.5 and 9%; LH, 0.05 U/liter and 10.7 and 13.0%; SHBG, 6.25 nmol/liter and 4 and 6%; PSA, 0.01 ng/ml and 5.0 and 6.4%; estradiol, 2.5 pg/ml (9.3 pmol/liter) and 5 and 8%; estrone, 2.5 pg/ml (9.3 pmol/liter) and 6 and 8%, respectively (23).

Statistical analyses

All variables were examined for distribution characteristics to assure that they met assumptions of normality and homogeneity of variance. We compared mean MHC+ myotube area and adipocyte number using one-way ANOVA. If a primary test of equality of means revealed significant treatment effect, intergroup comparisons were performed using the Tukey-Kramer procedure.

For studies in hypogonadal men, baseline and 12-wk hormone levels, FFM, muscle strength, and other outcomes were compared using Student's paired *t* test. *P* values < 0.05 were considered significant. A sample size of nine subjects was based on the assumption that 4-androstenedione administration would increase testosterone levels by an average 150 ng/dl with SD of 100 ng/dl (effect size, 1.5), FFM by 1.5 kg with SD of 1.5 kg (effect size, 1), and leg press strength by 10 kg with SD of 10 kg (effect size, 1). Using paired *t* test, a sample of nine subjects provided 90% power to detect 150-ng/dl increase in testosterone and greater than 80% power to detect 1.5-kg increase in FFM and 10-kg increase in leg press strength.

Results

4-Androstenedione binds AR-LBD with a lower affinity than DHT

In fluorescence polarization assay, graded concentrations of 4-androstenedione and DHT displaced FA from the binding pocket of AR-LBD dose dependently (Fig. 1A). Dissociation constants (K_d) of DHT and 4-androstenedione were calculated as 10 ± 0.4 and 648 ± 21 nM, respectively.

4-Androstenedione promotes AR nuclear translocation in C3H10T1/2 pluripotent cells

AR immunostaining was localized in the cytoplasm of C3H10T1/2 cells treated with medium alone (Fig. 1B). Treatment with 10 nM DHT or 100 nM 4-androstenedione caused AR immunostaining to be detected in nuclei, consistent with its nuclear translocation.

4-Androstenedione stimulates myogenic differentiation in C3H10T1//2 pluripotent cells

We have shown that incubation of azacytidine-treated, C3H10T1/2 cells with testosterone or DHT increases MHC+ myotube area and up-regulates myogenic markers, MHC, and MyoD (20). MHC expression was observed in multinucleated myotubes and was significantly increased in a concentration-dependent manner in cells treated with 4-androstenedione or DHT (Fig. 2A). Quantitative image analysis (Fig. 2A, *lower* panel) showed that 4-androstenedione increased MHC+ myotube area dose dependently. Pregnenolone (200 nM), a steroid with little androgenic activity, had no significant effect on MHC+ myotube area, indicating that these effects are not common to all steroid hormones (not shown).

MyoD protein, a transcription factor essential for myogenic differentiation, was up-regulated by androstenedione (Fig. 2C).

Wells treated with 1 μM [23.8 ± 1.6 adipocytes per high-power field (hpf)] and 3 μM (15.7 ± 1.3 adipocytes per hpf) 4-androstenedione and 10 nM DHT (23.7 ± 1.6 adipocytes per hpf) had fewer adipocytes than controls (38.9 ± 4.5 adipocytes per hpf; $P < 0.001$ for each comparison); effects of 1 μM 4-androstenedione were blocked by bicalutamide (33.5 ± 1.8 adipocytes per hpf; *P* value not significant *vs.* control).

Effects of 4-androstenedione on myogenesis are blocked by an AR antagonist

Graded doses of 4-androstenedione or 10 nM DHT resulted in higher MHC+ myotube area than medium alone ($P < 0.001$ for each comparison); the effects of 100 nM 4-androstenedione were inhibited by 100 and 500 nM bicalutamide ($P < 0.0001$ *vs.* 100 nM 4-androstenedione alone) (Fig. 2B). Stimulation of MyoD protein (Fig. 2C) by 4-androstenedione was also blocked by bicalutamide.

Effects of 4-androstenedione administration in hypogonadal men

Baseline characteristics of participants. Nine eligible hypogonadal men were started on treatment; eight completed 3 months of treatment. Seven men had hypogonadotropic hypogonadism and two hypergonadotropic hypogonadism (Table 1).

Compliance. Mean compliance, assessed by counting unused capsules, was 92% (range, 75–100%).

Hormonal responses (Fig. 3 and Table 2). 4-Androstenedione administration was associated with a significant increase in circulating androstenedione levels ($+4.6 \pm 0.7$ ng/ml [16.0 nmol/liter]; $P = 0.004$). $C_{average}$ total and free testosterone (Fig. 3), estradiol, and estrone levels (Table 2) were signifi-

TABLE 1. Baseline characteristics of the participants

Diagnosis	Age (yr)	Height (cm)	Weight (kg)	T (ng/dl)	LH (U/liter)
Kallmann's syndrome	36	178.0	92.3	10	0.5
Idiopathic hypogonadotropic hypogonadism	59	178.0	88.2	257	5.2
Hypogonadotropic hypogonadism after TSS	45	184.1	94.7	108	2.9
Klinefelter's syndrome	65	170.5	69.6	41	11.7
Idiopathic hypogonadotropic hypogonadism	50	175.0	101.4	256	2.3
Hypogonadotropic hypogonadism after TSS	27	155.0	68.2	271	1.3
Klinefelter's syndrome	57	196.1	114.0	49	13.4
Klinefelter's syndrome	36	181.2	117.3	84	20.2
Kallmann's syndrome	36	167.6	98.2	16	0.1

To convert serum testosterone concentrations (T) from ng/dl to nmol/liter, multiply the serum concentrations in ng/dl by 0.03467. TSS, Transsphenoidal surgery.

cantly higher on d 84 than at baseline. Serum androstenedione, total and free testosterone (Fig. 3), estradiol, and estrone levels (not shown) were higher throughout the 8-h sampling period on d 84 than on d 0. On d 84, mean androstenedione levels increased further after 4-androstenedione administration, reaching a peak at 4 h and returning to baseline by 8 h. SHBG concentrations decreased significantly after treatment (–18 ± 3 nmol/liter; $P = 0.001$).

Body composition (Table 3). FFM (+1.7 ± 0.5 kg; $P = 0.012$) and lean body mass (LBM) (+1.6 ± 0.5 kg; $P = 0.018$) increased significantly after 4-androstenedione treatment. Gains in LBM were distributed evenly between appendicular (+0.6 ± 0.3 kg; $P = 0.027$) and truncal (+0.7 ± 0.3 kg; $P = 0.044$) compartments. There were no significant changes in fat mass ($P = 0.432$) or percent body fat ($P = 0.066$).

Muscle strength (Table 3). Maximal voluntary muscle strength increased significantly in bench press (+4.3 kg; $P = 0.006$) and leg press exercises (+18.8 kg; $P = 0.045$).

Safety markers (Table 4). 4-Androstenedione administration was associated with a significant decrease in high-density lipoprotein (HDL) cholesterol (–12 ± 3 mg/dl; $P = 0.003$). Total and low-density lipoprotein (LDL) cholesterol, triglyceride, PSA, hemoglobin, hematocrit, aspartate aminotransferase, and alanine aminotransferase did not change significantly.

Discussion

4-Androstenedione possesses many essential properties of an androgen: it binds the LBD of AR with a lower affinity

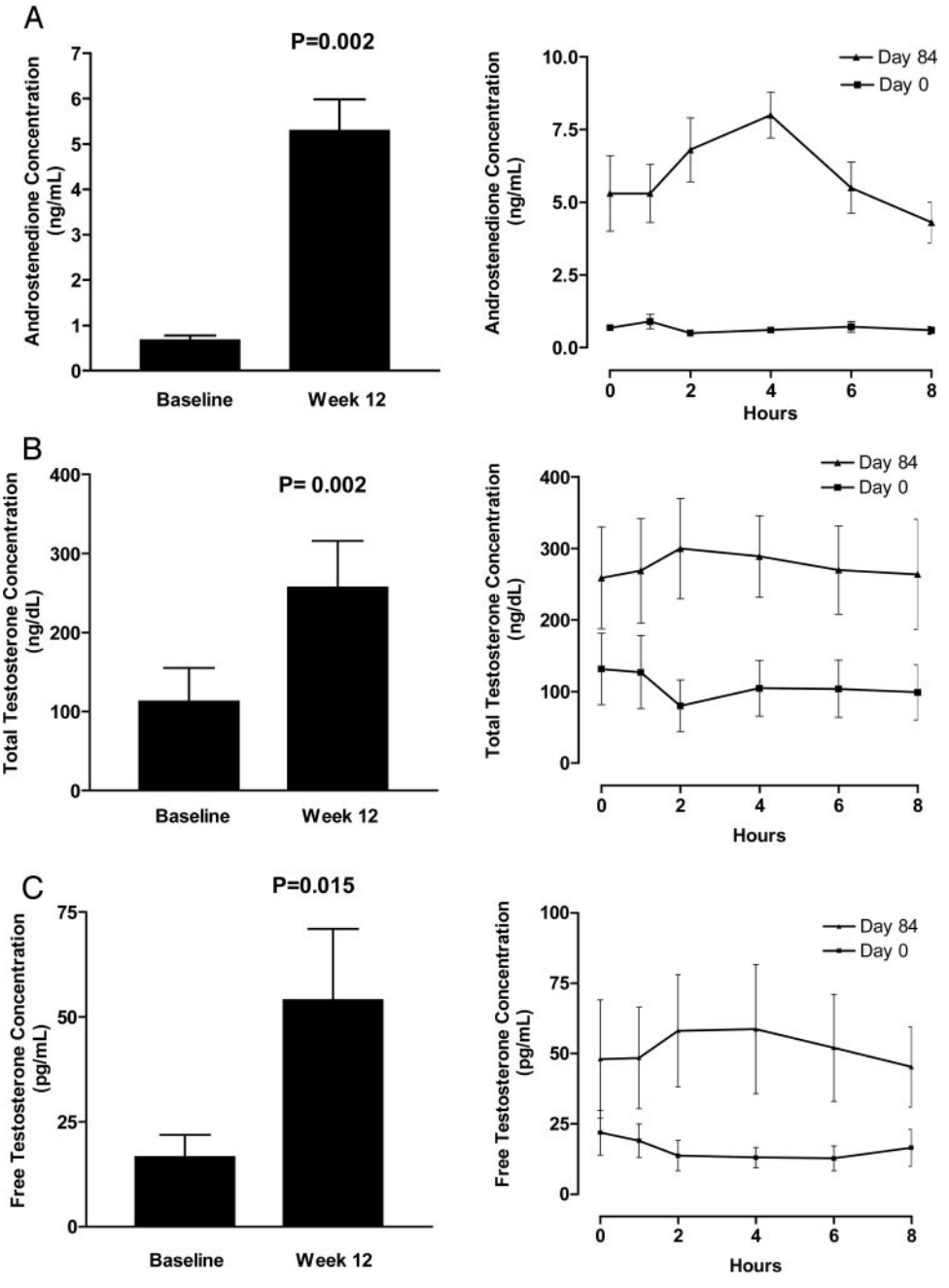

FIG. 3. The effects of androstenedione administration on serum androstenedione, total and free testosterone, estradiol, and estrone levels in healthy, hypogonadal men. A, The *left panel* shows $C_{average}$ serum androstenedione (mean ± SEM) levels before and after 12 wk of treatment with 500 mg androstenedione administered thrice daily to eight healthy, hypogonadal men. The *right panel* shows serum androstenedione (mean ± SEM) levels over an 8-h period after administration of 500 mg androstenedione immediately at time 0. To convert androstenedione concentrations from ng/ml to nmol/liter, multiply the concentrations in ng/ml by 3.49. B, Serum $C_{average}$ total testosterone (mean ± SEM) levels before and after 12 wk of treatment with 500 mg androstenedione administered thrice daily to eight healthy, hypogonadal men (*left panel*). The *right panel* shows serum total testosterone levels (mean ± SEM) over an 8-h period after administration of 500 mg androstenedione. To convert serum testosterone concentrations from ng/dl to nmol/liter, multiply serum concentrations in ng/dl by 0.0347. C, Serum $C_{average}$ free testosterone (mean ± SEM) levels before and after 12 wk of treatment with 500 mg androstenedione administered thrice daily to eight healthy, hypogonadal men (*left panel*). The *right panel* shows serum free testosterone levels (mean ± SEM) over an 8-h period after administration of 500 mg androstenedione. To convert serum free testosterone concentrations from pg/ml to pmol/liter, multiply the serum concentrations in pg/ml by 3.47.

TABLE 2. Effect of androstenedione administration on serum estradiol, estrone, and SHBG levels

Hormone	Baseline	Wk 12	Change from baseline	*P* value
Estradiol (pg/ml)	20.4 ± 2.5	40.0 ± 5.0	19.5 ± 3.5	0.0008
Estrone (pg/ml)	29.1 ± 6.7	89.0 ± 27.0	59.9 ± 32.7	0.029
SHBG (nmol/liter)	33.2 ± 5	16 ± 4	−18 ± 3	0.001

Serum $C_{average}$ estradiol, estrone, and SHBG (mean ± SEM) levels before and after 12 wk of treatment with 500 mg androstenedione administered thrice daily to eight healthy, hypogonadal men. To convert estradiol concentrations from pg/ml to pmol/liter, multiply the concentration in pg/ml by 3.67. To convert estrone concentrations from pg/ml to pmol/liter, multiply the concentration in pg/ml by 3.70.

than DHT, induces AR-nuclear translocation, and promotes myogenic differentiation and inhibits adipogenic differentiation of mesenchymal pluripotent cells, which have been shown previously to respond to androgens by an increase in myogenesis (20, 27). Promyogenic effects of 4-androstenedione in this bioassay are blocked by bicalutamide, an AR antagonist, indicating that these effects are mediated through an AR pathway. Administration of 4-androstenedione to hypogonadal men is associated with significant increments in serum total and free testosterone concentrations, whole-body and appendicular lean mass, and muscle strength. 4-Androstenedione's low AR binding affinity and substantially lesser promyogenic potency than DHT in C3H10T1/2 cells indicate that it is a weak androgen and that its anabolic effects of 4-androstenedione in humans at the 1500-mg daily dose are likely mediated through its conversion to testosterone.

The manufacturers of androstenedione have resisted efforts to regulate its sales by citing the lack of evidence of its *in vivo* anabolic or androgenic activity. In addition to the experimental data presented in this manuscript, several experiments of nature provide additional evidence of androgenic properties of 4-androstenedione. Females of several mammalian species, including moles and spotted hyenas (*Crocuta crocuta*) are masculinized because of raised androstenedione levels (17, 19, 29, 30). Female spotted hyenas have higher androstenedione levels, are heavier and more aggressive than males, and possess a large, erectile pseudopenis (29–30). Testosterone levels in female spotted hyenas are lower than in male counterparts and do not account for the male-like characteristics of the female. 4-Androstenedione is a potent organizer of male anatomical and behavioral differentiation in rodents (30). Thus, 4-androstenedione is an androgen that when present in sufficient concentrations virilizes female spotted hyenas. These experiments of nature, when viewed together with our data, illustrate the androgenic-anabolic properties of androstenedione and contradict specious arguments to the contrary put forth by manufacturers of dietary supplements.

Our study differed in several aspects from previous studies that evaluated the effects of 4-androstenedione administration in men (7, 9, 12, 15, 31). Combined application of *in vitro* and *in vivo* approaches allowed us to establish androgenic properties of 4-androstenedione by using several independent criteria. We used higher doses of 4-androstenedione for a longer duration than had been used in previous human studies (32–34); the 1500-mg daily dose is similar to doses used by many athletes (3, 4). In contrast to previous studies in eugonadal men, performance of this study in hypogonadal men enabled us to demonstrate sustained increments in testosterone levels. We do not know whether similar increments in testosterone levels would be observed in eugonadal men after 4-androstenedione administration because of suppression of endogenous testosterone production. Akin to previous androgen studies in hypogonadal men (35–39), we did not include a placebo group because of ethical constraints; the Food and Drug Administration has approved the use of several androgen formulations based on results of open-label trials of testosterone replacement in hypogonadal men (35, 40).

We do not know what fraction of muscle mass gain during 4-androstenedione administration is a result of increased androstenedione levels and what fraction is a result of its conversion to testosterone. Androstenedione is a weak androgen by itself, and its circulating concentrations are substantially lower than its K_d (648 nM), suggesting that its anabolic effects in hypogonadal men, at the 1500-mg dose, are likely largely because of its conversion to testosterone. This situation is not dissimilar from that of prednisone, a glucocorticoid, and some androgenic steroids that also require conversion in the body to more active metabolites.

FFM and muscle strength gains observed with 4-androstenedione administration are similar in magnitude to those reported in studies of testosterone replacement in hypogonadal men using transdermal testosterone systems (35, 37, 40). Administration of 6 mg testosterone daily by a scrotal patch increased LBM by an average 1.9 kg (37), whereas nominal delivery of 10 mg testosterone daily by a transdermal gel for 90 d increased LBM by 1.5 kg (41). Another study (36), using 5 mg sublingual testosterone thrice daily for 6 months, reported 0.9 kg gain in FFM and 8.7 kg gain in leg press strength. A testosterone gel trial reported 11–13 kg gain in leg press strength (35). Therefore, it is remarkable that

TABLE 3. Effects of androstenedione on body composition, muscle strength, and biochemical indices

	Baseline	Wk 12	Change	*P*
Body composition measures				
Whole-body LBM (kg)	62.4 ± 3.7	64.0 ± 3.5	1.6 ± 0.5	0.018
Whole-body FFM (kg)	64.8 ± 3.8	66.5 ± 3.6	1.7 ± 0.5	0.012
Appendicular LBM (kg)	28.8 ± 2.4	29.4 ± 2.3	0.6 ± 0.3	0.027
Trunk LBM (kg)	29.7 ± 1.5	30.4 ± 1.3	0.7 ± 0.3	0.044
Total body fat (kg)	30.1 ± 3.2	29.6 ± 3.1	−0.5 ± 0.6	0.432
Percentage body fat (%)	31.3 ± 1.5	30.4 ± 1.6	−0.8 ± 0.3	0.066
Muscle strength measures				
Leg press strength (kg)	261.9 ± 41.7	280.7 ± 39.7	18.8 ± 17.3	0.045
Bench press strength (kg)	46.0 ± 9.7	50.3 ± 12.4	4.3 ± 3.1	0.006

TABLE 4. Safety markers

	Baseline	Wk 12	Change	*P*
Plasma lipids				
HDL-C (mg/dl)	44 ± 4	33 ± 2	−12 ± 3	0.003
LDL-C (mg/dl)	135 ± 6	137 ± 10	2 ± 10	0.848
Total cholesterol (mg/dl)	205 ± 6	191 ± 11	−13 ± 11	0.279
Other biochemical indices				
PSA (ng/ml)	0.4 ± 0.1	0.4 ± 0.1	−0.0 ± 0.1	0.906
Hematocrit (liter/liter)	0.41 ± 0.20	0.42 ± 0.20	0.02 ± 0.01	0.851

To convert cholesterol, HDL cholesterol (HDL-C), and LDL cholesterol (LDL C) concentrations from mg/dl to mmol/liter, multiply concentrations in mg/dl by 0.02586.

testosterone is a controlled substance, whereas 4-androstenedione had been sold over the counter until recently.

Although 4-androstenedione inhibited adipogenic differentiation of mesenchymal, pluripotent cells *in vitro*, its administration to hypogonadal men was not associated with significant reduction in whole-body fat mass. This might have been related to the relatively short treatment duration; several previous testosterone replacement studies in hypogonadal men of 10–12 wk duration also failed to reveal significant changes in fat mass. Adipocytes have a long half-life, and longer treatment duration in a larger number of subjects might be required to demonstrate significant effects of 4-androstenedione on fat mass.

Plasma HDL cholesterol decreased and estradiol and estrone levels increased during 4-androstenedione administration. Altered testosterone-to-estrogen ratio might induce gynecomastia. Experts have expressed concern about potential cytogenetic effects of environmental estrogens on sperm (28); we do not know whether long-term exposure to increased estrogen levels during 4-androstenedione use might adversely affect semen quality, inflammation markers, and cardiovascular risk.

A compound must have structural and pharmacological resemblance to testosterone and promote muscle growth to be classified as an anabolic steroid (5). 4-Androstenedione has remarkable structural similarity to testosterone, binds AR, promotes myogenic differentiation through an AR-mediated mechanism, and increases muscle mass and strength in humans. Therefore, 4-androstenedione is a *bona fide* androgen with anabolic properties that meets the essential criteria for an anabolic steroid established by the Controlled Substance Act (5).

Acknowledgments

Received August 10, 2004. Accepted October 27, 2004.

Address all correspondence and requests for reprints to: Shalender Bhasin, M.D., Professor of Medicine, UCLA School of Medicine, Director, Drew-UCLA Reproductive Science Research Center, Chief, Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, California 90059. sbhasin@ucla.edu.

This work was supported by Grants 1RO1HD043348-01 and U54HD041748-01, Research Center at Minority Institutions Grants P20RR11145 and G12RR03026, and a University of California AIDS Research Program DrewCares HIV Center grant.

References

1. **Kottke MK** 1998 Scientific and regulatory aspects of nutraceutical products in the United States. Drug Dev Ind Pharm 24:1177–1195
2. **Stevens K** 1995 Dietary Supplement Health and Education Act: quiet but far-reaching consequences. Altern Ther Health Med 1:17–18
3. **Yesalis CE, Barsukiewicz CK, Kopstein AN, Bahrke MS** 1997 Trends in anabolic-androgenic steroid use among adolescents. Arch Pediatr Adolesc Med 151:1197–1206
4. **Yesalis 3rd CE** 1999 Medical, legal, and societal implications of androstenedione use. JAMA 281:2043–2044
5. **Anabolic Steroid Control Act PLN-, XIX** 1990 Anabolic Steroid Control Act, P.L.N. 1990; XIX:104 Stat 4851
6. **King DS, Sharp RL, Vukovich MD, Brown GA, Reifenrath TA, Uhl NL, Parsons KA** 1999 Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomized controlled trial. JAMA 281:2020–2028
7. **Rasmussen BB, Volpi E, Gore DC, Wolfe RR** 2000 Androstenedione does not stimulate muscle protein anabolism in young healthy men. J Clin Endocrinol Metab 85:55–59
8. **Broeder CE, Quindry J, Brittingham K, Panton L, Thomson J, Appakondu S, Breuel K, Byrd R, Douglas J, Earnest C, Mitchell C, Olson M, Roy T, Yarlagadda C** 2000 The Andro Project: physiological and hormonal influences of androstenedione supplementation in men 35 to 65 years old participating in a high-intensity resistance training program. Arch Intern Med 160:3093–3104
9. **Leder BZ, Longcope C, Catlin DH, Ahrens B, Schoenfeld DA, Finkelstein JS** 2000 Oral androstenedione administration and serum testosterone concentrations in young men. JAMA 283:779–782
10. **Brown GA, Vukovich MD, Reifenrath TA, Reifenrath TA, Uhl NL, Parsons KA, Sharp RL, King DS** 2000 Effects of anabolic precursors on serum testosterone concentrations and adaptations to resistance training in young men. Int J Sport Nutr Exerc Metab 10:340–359
11. **Brown GA, Vukovich MD, Martini ER, Kohut ML, Franke WD, Jackson DA, King DS** 2000 Endocrine responses to chronic androstenedione intake in 30- to 56-year-old men. J Clin Endocrinol Metab 85:4074–4080
12. **Ballantyne CS, Phillips SM, MacDonald JR, Tarnopolsky MA, MacDougall JD** 2000 The acute effects of androstenedione supplementation in healthy young males. Can J Appl Physiol 25:68–78
13. **Mahesh VB, Greenblatt RB** 1962 The in vivo conversion of dehydroepiandrosterone and androstenedione to testosterone in the human. Acta Endocrinol 41:400–406
14. **Beckham SG, Earnest CP** 2003 Four weeks of androstenedione supplementation diminishes the treatment response in middle aged men. Br J Sports Med 37:212–218
15. **Earnest CP, Olson MA, Broeder CE, Breuel KF, Beckham SG** 2001 In vivo 4-androstene-3,17-dione and 4-androstene-3β,17β-diol supplementation in young men. Eur J Appl Physiol 81:229–232
16. **Spratt DI, Pont A, Marcus R,** Changes in serum sex-steroid concentrations following oral administration of androstenedione. Program of the 81st Annual Meeting of The Endocrine Society, San Diego, CA, 1999, p 64 (Abstract OR2-3)
17. **Frank L, Weldele M, Glickman S** 1995 Fitness costs of genital masculinization in female spotted hyenas. Nature 377:584–585
18. **Frank L** 1995 Evolution of genital masculinization: why do female hyaenas have such a large 'penis'? Trends Ecol Evol 12:58–62
19. **Goymann W, East M, Hofer H** 2001 Androgens and the role of female "hyperaggressiveness" in spotted hyenas (*Crocuta crocuta*). Horm Behav 39:83–92
20. **Singh R, Artaza JN, Taylor WE, Gonzalez-Cadavid NF, Bhasin S** 2003 Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. Endocrinology 144:5081–5088
21. **Ozers M, Hill J, Errin K, Wood JR, Nardulli AM, Royer CA, Gorski J** 1997 Equilibrium binding of estrogen receptor using fluorescence anisotropy. Nucleic Acid Res 272:30405–30411
22. **Bhasin S, Storer TW, Berman N, Yarasheski KE, Clevenger B, Phillips J, Lee WP, Bunnell TJ, Casaburi R** 1997 Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. J Clin Endocrinol Metab 82:407–413
23. **Bhasin S, Storer TW, Berman N, Callegari C, Clevenger B, Phillips J, Bunnell TJ, Tricker R, Shirazi A, Casaburi R** 1996 The effects of supraphysiologic doses

of testosterone on muscle size and strength in normal men. N Engl J Med 335:1–7
24. **Woodhouse LJ, Gupta N, Bhasin M, Singh AB, Ross R, Phillips J, Bhasin S** 2004 Dose-dependent effects of testosterone on regional adipose tissue distribution in healthy young men. J Clin Endocrinol Metab 89:718–726
25. **Storer TW, Magliano L, Woodhouse L, Lee ML, Dzekov C, Dzekov J, Casaburi R, Bhasin S** 2003 Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension. J Clin Endocrinol Metab 88:1478–1485
26. **Sinha-Hikim I, Arver S, Beall G, Shen R, Guerrero M, Sattler F, Shikuma C, Nelson JC, Landgren BM, Mazer NA, Bhasin S** 1998 The use of a sensitive equilibrium dialysis method for the measurement of free testosterone levels in healthy, cycling women and in human immunodeficiency virus-infected women . J Clin Endocrinol Metab [Erratum (1998) 83:2959] 83:1312–1318
27. **Bhasin S, Taylor WE, Singh R, Artaza J, Sinha-Hikim I, Jasuja R, Choi H, Gonzalez-Cadavid NF** 2003 The mechanisms of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action. J Gerontol A Biol Sci Med Sci 58:M1103–M1110
28. **McLachlan JA** 2001 Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr Rev 22:319–341
29. **Glickman S, Frank L, Pavgi S, Licht P** 1992 Hormonal correlates of 'masculinization' in female spotted hyaenas (*Crocuta crocuta*). 1. Infancy to sexual maturity. J Reprod Fertil 95:451–462
30. **Glickman S, Frank L, Davidson J, Smith E, Siiteri P** 1987 Androstenedione may organize or activate sex-reversed traits in female spotted hyenas. Proc Natl Acad Sci USA 84:3444–3447
31. **King DS, Sharp RL, Vukovich MD, Brown GA, Reifenrath TA, Uhl NL, Parsons KA** 1999 Effect of oral androstenedione on serum testosterone and adaptations to resistance training in young men: a randomized controlled trial. JAMA 281:2020–2028
32. **Kicman AT, Bassindale T, Cowan DA, Dale S, Hutt AJ, Leeds AR** 2003 Effect of androstenedione ingestion on plasma testosterone in young women: a dietary supplement with potential health risks. Clin Chem 49:167–169
33. **Brown GA, Dewey JC, Brunkhorst JA, Vukovich MD, King DS** 2004 Changes in serum testosterone and estradiol concentrations following acute androstenedione ingestion in young women. Horm Metab Res 36:62–66
34. **Leder BZ, Leblanc KM, Longcope C, Lee H, Catlin DH, Finkelstein JS** 2002 Effects of oral androstenedione administration on serum testosterone and estradiol levels in postmenopausal women. J Clin Endocrinol Metab 87:5449–5454
35. **Wang C, Swedloff RS, Iranmanesh A, Dobs A, Snyder PJ, Cunningham G, Matsumoto AM, Weber T, Berman N** 2000 Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameters in hypogonadal men: Testosterone Gel Study Group. J Clin Endocrinol Metab 85:2839–2853
36. **Wang C, Eyre DR, Clark R, Kleinberg D, Newman C, Iranmanesh A, Veldhuis J, Dudley RE, Berman N, Davidson T, Barstow TJ, Sinow R, Alexander G, Swerdloff RS** 1996 Sublingual testosterone replacement improves muscle mass and strength, decreases bone resorption, and increases bone formation markers in hypogonadal men: a clinical research center study. J Clin Endocrinol Metab 81:3654–3662
37. **Snyder PJ, Peachey H, Berlin JA, Hannoush P, Haddad G, Dlewati A, Santanna J, Loh L, Lenrow DA, Holmes JH, Kapoor SC, Atkinson LE, Strom BL** 2000 Effects of testosterone replacement in hypogonadal men. J Clin Endocrinol Metab 85:2670–2677
38. **Brodsky IG, Balagopal P, Nair KS** 1996 Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men: a clinical research center study. J Clin Endocrinol Metab 81:3469–3475
39. **Katznelson L, Finkelstein JS, Schoenfeld DA, Rosenthal DI, Anderson EJ, Klibanski A** 1996 Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. J Clin Endocrinol Metab 81:4358–4365
40. **Steidle C, Schwartz S, Jacoby K, Sebree T, Smith T, Bachand R** 2003 AA2500 testosterone gel normalizes androgen levels in aging males with improvements in body composition and sexual function. J Clin Endocrinol Metab 88:2673–2681
41. **McNicholas TA, Dean JD, Mulder H, Carnegie C, Jones NA** 2003 A novel testosterone gel formulation normalizes androgen levels in hypogonadal men, with improvements in body composition and sexual function. BJU Int 91:69–74

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.