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Androgens Stimulate Myogenic Differentiation and Inhibit Adipogenesis in C3H 10T1/2 Pluripotent Cells through an Androgen Receptor-Mediated Pathway

RAJAN SINGH, JORGE N. ARTAZA, WAYNE E. TAYLOR, NESTOR F. GONZALEZ-CADAVID, AND SHALENDER BHASIN

Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University of Medicine and Science, Los Angeles, California 90059

Testosterone supplementation increases skeletal muscle mass and decreases fat mass; however, the underlying mechanisms are unknown. We hypothesized that testosterone regulates body composition by promoting the commitment of mesenchymal pluripotent cells into myogenic lineage and inhibiting their differentiation into adipogenic lineage. Mouse C3H 10T1/2 pluripotent cells were treated with testosterone (0–300 nM) or dihydrotestosterone (DHT, 0–30 nM) for 0–14 d, and myogenic conversion was evaluated by immunocytochemical staining for early (MyoD) and late (myosin heavy chain II; MHC) myogenic markers and by measurements of MyoD and MHC mRNA and protein. Adipogenic differentiation was assessed by adipocyte counting and by measurements of peroxisomal proliferator-activated receptor γ 2 (PPARγ2) mRNA and PPARγ2 protein and CCAAT/enhancer binding protein α. The number of MyoD+ myogenic cells and MHC+ myotubes and MyoD and MHC mRNA and protein levels increased dose dependently in response to testosterone and DHT treatment. Both testosterone and DHT decreased the number of adipocytes and down-regulated the expression of PPARγ2 mRNA and PPARγ2 protein and CCAAT/enhancer binding protein α. Androgen receptor mRNA and protein levels were low at baseline but increased after testosterone or DHT treatment. The effects of testosterone and DHT on myogenesis and adipogenesis were blocked by bicalutamide. Therefore, testosterone and DHT regulate lineage determination in mesenchymal pluripotent cells by promoting their commitment to the myogenic lineage and inhibiting their differentiation into the adipogenic lineage through an androgen receptor-mediated pathway. The observation that differentiation of pluripotent cells is androgen dependent provides a unifying explanation for the reciprocal effects of androgens on muscle and fat mass in men. (*Endocrinology* 144: 5081–5088, 2003)

TESTOSTERONE IS AN important determinant of body composition in male mammals (1, 2). Androgen deficiency is associated with decreased muscle mass and increased fat mass (3). Conversely, testosterone supplementation increases fat-free mass (4–7) and decreases fat mass (6–7) in hypogonadal men, HIV-infected men with weight loss (8), and older men with low testosterone concentrations (9–11). Testosterone's effects on muscle and fat mass are related to its dose (12–13). Not surprisingly, some athletes take large amounts of androgens to increase muscle mass and strength (1). Testosterone administration is associated with hypertrophy of both type I and II muscle fibers (14) and significant increases in myonuclear and satellite cell numbers (15).

The mechanisms by which testosterone regulates body composition are poorly understood. The prevalent view is that testosterone increases muscle mass by stimulating muscle protein synthesis (5, 16–19). Indeed, testosterone administration increases nitrogen retention in castrated male rats (16), in eunuchoidal men, in women, and in boys before puberty (17). Testosterone administration to young, hypogonadal men (5) and older men with low testosterone levels stimulates muscle protein synthesis (18–19). The protein synthesis hypothesis, however, does not explain the decrease in fat mass and the increase in myonuclear and satellite cell numbers associated with testosterone administration.

Muscle growth and regeneration during postnatal development and hypertrophy is dependent on the addition of myonuclei to muscle fibers (20–21). Because the nuclei within the muscle fibers are post mitotic, new myonuclei must be contributed by the satellite cells (20–22). An increase in satellite cell number is an antecedent of an increase in myonuclear number and muscle fiber hypertrophy (20–22). Testosterone supplementation increases satellite cell number in the levator ani of rats (23) and the skeletal muscle of men (15). The uncommitted, pluripotent stem cells of mesodermal origin that are resident within the muscle serve as reservoirs for the generation of new satellite cells or myoblasts during muscle regeneration or hypertrophy (24) and of adipocytes in the muscle and adipose deposits throughout the body (25). To explain the reciprocal changes in fat and muscle mass and the increase in satellite cell number during testosterone administration, we hypothesized that, in addition to direct effects on protein synthesis and satellite cell replication, testosterone promotes the commitment of pluripotent precursor cells into the myogenic lineage and inhibits their differentiation into the adipogenic lineage.

To test this hypothesis, we used pluripotent, mesenchymal C3H 10T1/2 (10T1/2) cells that are capable of differentiating into muscle, fat, cartilage, and bone cells and that have been

Abbreviations: AR, Androgen receptor; C/EBPα, CCAAT/enhancer binding protein α; DHT, dihydrotestosterone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MHC, myosin heavy chain II; PPARγ2, peroxisomal proliferator-activated receptor γ 2.

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used widely as a model for studying the regulation of myogenic and adipogenic lineage determination (26–28). Commitment of 10T1/2 cells to myogenic lineage is associated with early activation of muscle-specific transcription factors, such as MyoD, myogenin, and Myf5, followed by the expression of desmin and myosin heavy chain II (MHC) in terminally differentiated cells (26–31). Peroxisomal proliferator-activated receptor γ 2 (PPARγ2) and CCAAT/enhancer binding protein α (C/EBPα) are key transcription factors necessary for adipogenic differentiation (31–33). We determined the effects of testosterone and dihydrotestosterone (DHT), two potent androgens, on the differentiation of 10T1/2 into myogenic and adipogenic lineages. We also assessed the effects of testosterone and DHT on the expression of myogenic and adipogenic differentiation markers. We evaluated whether these effects occur through an androgen receptor (AR)-mediated pathway.

Materials and Methods

Tissue culture

Mouse 10T1/2 cells, grown in DMEM with 10% fetal bovine serum growth medium at 37 C, were treated with 20 μM 5-azacytidine for 3 d, split 1:2, allowed to recover for 2 d, and seeded at 70% confluence in six-well plates or chamber slides and grown with test agents for 0–14 d.

Immunocytochemical analyses of MHC, MyoD, and AR

Cells grown in eight-well chamber slides were fixed in 2% paraformaldehyde, quenched with H_2O_2, blocked with normal horse serum, and incubated with a specific antibody. Detection was based on a secondary biotinylated antibody (1:200), followed by the addition of the streptavidin-horseradish peroxidase ABC complex (1:100; Vectastain Elite ABC System, Novocastra Laboratories, Newcastle upon Tyne, UK) and 3,3′-diaminobenzidine. The cells were counterstained with Meyer's hematoxylin. In negative controls, we either omitted the first antibody or used a rabbit nonspecific IgG.

The cytochemical staining was quantitated by densitometry using the ImagePro program (Media Cybernetics, Silver Spring, MD). The number of MyoD- and MHC-positive cells was counted against the total number of cells determined by counterstaining. The area of MHC+ cells was computed per field and averaged over 10 fields.

Oil red O staining and adipocyte counting

10T1/2 cells were washed, fixed in 2% paraformaldehyde, and stained with 0.3% Oil red O. The number of adipocytes was counted under a bright-field microscope in ten 100-X fields and averaged.

Western blot analysis

Cell lysates (50–100 μg) were subjected to Western blot analyses by 7.5% gel electrophoresis, using 1:200 mouse monoclonal anti-MHC (slow, Vector Laboratories, Inc., Burlingame, CA), 1:500 anti-AR, 1:500 anti-MyoD, 1:1000 anti-PPARγ, 1:300 anti-C/EBPα, or 1:10000 antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The washed filters were incubated with 1:1000 dilution of secondary antibodies linked to horseradish peroxidase. Immunoreactive bands were visualized by using the enhanced chemiluminescence detection system (Amersham, Chicago, IL).

RT-PCR and real-time quantitative PCR

Total RNA, extracted by using the Trizol-Reagent (Invitrogen, Carlsbad, CA), was reverse transcribed, and cDNA was amplified for 36 cycles by PCR at 94 C for 30 sec, primer annealing at 58 C for 30 sec, and extension at 72 C for 1 min. PCR products were analyzed in 2% agarose gels. The locations of the forward/reverse PCR primers are as follows: AR (187 bp) 1937-1958/2124-2102 on S56585 and GAPDH (152 bp) 606-626/758-738 on BC023196.

For real-time RT-PCR analysis of mRNA levels, forward/reverse primers were as follows: MyoD (130 bp) 801-821/930-911 on M84918 and PPARγ2 (241 bp) 79-99/320-299 on BC021798. The primers for PPARγ2 were specific for PPARγ2 and did not detect PPARγ1. The Qiagen Syber-green RT-PCR kit with HotStar Taq DNA polymerase was used (Qiagen, Valencia, CA) with the iCycler PCR thermocycler and fluorescent detector lid (Bio-Rad, Hercules, CA). The protocol included RT for 30 min at 50 C, using 250 ng (25 ng for GAPDH) RNA; melting for 15 min at 95 C; and 40 cycles of three-step PCR, including melting for 15 sec at 95 C, annealing for 30 sec at 58 C, and elongation for 30 sec at 72 C with a detection step of 15 sec at 81 C; followed by a melting curve from 55–95 C at the rate of 0.5 C per 10 sec. The inverse derivatives of melting curves showed sharp peaks for MyoD product at 88 C, PPARγ2 at 83.5 C, and GAPDH at 86 C, indicating the correct products. Samples were analyzed in quadruplicate for MyoD or PPARγ2 in parallel with GAPDH control; standard curves were generated by log dilutions of pMyoD and pGAPDH plasmids or PPARγ2 PCR fragment from 1 fg to 1 ng. mRNA levels were calculated using the iCycler iQ software (Bio-Rad). The ratios of MyoD or PPARγ2 mRNA to GAPDH mRNA were computed.

Statistical analyses

All data are presented as mean ± SEM. Between-group differences were analyzed by using ANOVA. If overall ANOVA revealed significant differences, then pair-wise comparisons between groups were performed using Tukey's procedure. All comparisons were two-tailed, and P values less than 0.05 were considered statistically significant. The experiments were repeated two to four times, and data from representative experiments are shown.

Results

Low basal level of AR expression in 10T1/2 cells and induction of AR expression by testosterone and DHT

10T1/2 cells pretreated with 5-azacytidine and grown for 14 d in growth medium expressed low levels of AR mRNA and protein as assessed by RT-PCR and Western blot analysis (Fig. 1A). The AR mRNA and protein levels, corrected for GAPDH mRNA and protein expression, respectively, were higher in cells incubated with DHT and testosterone (data not shown) than in control cells (Fig. 1A). By immunocytochemical staining using a specific anti-AR antibody (Fig. 1B), very little staining (mostly cytoplasmic) was found in untreated control wells, and none was observed in cells that were not treated with anti-AR antibody (data not shown). Incubation with graded doses of DHT or testosterone increased nuclear AR staining (Fig. 1B shows only selective doses). Androgen stimulation of AR expression was blocked by coincubation with an AR antagonist, flutamide, suggesting that AR is involved in this autoregulation.

Testosterone and DHT stimulate myogenesis

MHC is expressed in terminally differentiated skeletal muscle cells and is a late marker of myogenic commitment. In preliminary experiments, we determined that by d 14, sufficient numbers of MHC+ myotubes were formed in untreated wells. Therefore, we used this time point to count the number of MHC+ myotubes and to evaluate changes in MHC expression. In contrast, MyoD expression was induced earlier, and we counted the number of MyoD+ cells at d 7. Figure 2A shows the dose-dependent effect of testosterone (0–300 nM) treatment on the area of MHC+ myogenic cells measured by immunocytochemistry. The expression of

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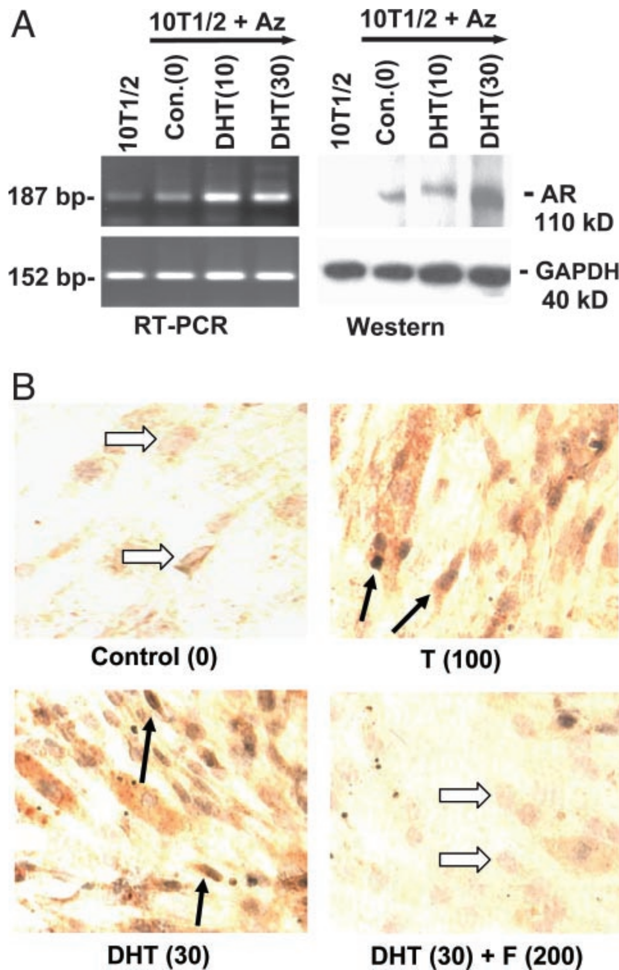

FIG. 1. Androgen induction of AR in 10T1/2 cells A, RT-PCR analysis of AR mRNA (*left*) and Western blots of AR protein (*right*). Cells were pretreated without (10T1/2) or with 5-azacytidine (Az) and grown for 14 d with medium [control (Con.)] or DHT (nM). Total RNA was analyzed by RT-PCR. Protein extracts were analyzed by Western blotting using anti-AR or anti-GAPDH antibody. B, Immunocytochemistry of AR protein. 10T1/2 cells were treated with medium alone, testosterone (T) (nM), DHT (nM), or DHT plus flutamide (F) and analyzed by immunocytochemistry using anti-AR antibody. *Solid arrows* point to AR+ nuclei, and *empty arrows* point to nuclei that did not stain with anti-AR antibody. No counterstain was used. Magnification, ×200.

MHC was observed mostly in multinucleated myotubes and was significantly increased in a concentration-dependent manner in cells treated with testosterone (Fig. 2A) or DHT (data not shown). In contrast, pregnenolone (200 nM), a steroid with little androgenic activity, had no significant effect on the number of MHC+ myotubes, indicating that these effects are not common to all steroid hormones. Quantitative image analysis of these MHC+ cells (Fig. 2A, *lower panel*) showed that treatment with graded concentrations of testosterone (0–300 nM) or DHT (0–30 nM) significantly increased the number of MHC+ myogenic cells; at the highest concentrations of testosterone and DHT, the area of MHC+ myotubes was increased 4- to 5-fold compared with control wells (mean: 135 myotubes per 10^3 μm^2 for testosterone *vs.* 26 for control, $P < 0.001$; and 104 myotubes per 10^3 μm^2 for DHT *vs.* 26 for control, $P < 0.001$). Western blot analysis confirmed that graded doses of testosterone dose-dependently increased MHC protein expression compared with vehicle control (Fig. 2B).

Testosterone up-regulated the expression of MyoD mRNA and protein; MyoD is a transcription factor that is essential for myogenic differentiation (Fig. 2, C and D). By real-time quantitative RT-PCR, the ratio of MyoD to GAPDH mRNA levels in cells treated with testosterone (30–300 nM) or DHT (3–30 nM; Fig. 2C) was significantly increased compared with controls. By Western blot analysis, DHT (30 nM) up-regulated MyoD protein expression by over 2-fold after normalizing for GAPDH (Fig. 2D).

Testosterone and DHT inhibit adipogenesis

10T1/2 cells were treated with testosterone or DHT for 12 d, and the number of differentiated adipocytes was counted (Fig. 3A). Testosterone and DHT each dose-dependently inhibited the number of adipocytes to approximately 50% of control at the highest androgen concentrations (mean: 18 adipocytes per ×100 field for testosterone *vs.* 35 for controls, $P = 0.0002$; 18 adipocytes per ×100 field for DHT *vs.* 33 for controls, $P = 0.0002$).

DHT and testosterone (data not shown) inhibited the expression of transcription factors that are important for adipogenic differentiation (Fig. 3). Thus, increasing testosterone (Fig. 3B) and DHT (data not shown) concentrations (0–300 nM) down-regulated the expression of PPARγ2 mRNA (measured by real-time RT-PCR). DHT (Fig. 3C) and testosterone (data not shown) dose-dependently inhibited the expression of PPARγ2 protein. DHT also inhibited the expression of 42- and 30-kDa C/EBPα proteins in a dose-dependent manner (Fig. 3C).

Effects of testosterone and DHT on myogenesis are blocked by an AR antagonist

To determine whether the effects of testosterone and DHT on myogenesis are mediated through the AR pathway, we assessed whether these effects could be blocked by bicalutamide, a competitive AR antagonist. Incubation of cells with 30 nM testosterone or 10 nM DHT resulted in a higher MHC+ myotube area than medium alone (Fig. 4, A and B). The effects of testosterone and DHT were inhibited dose dependently by bicalutamide. The stimulation of MHC protein by testosterone and DHT, by Western blotting, was also blocked by graded concentrations of bicalutamide (Fig. 4C).

Incubation of 10T1/2 cells with 30 nM testosterone and 10 nM DHT was associated with a greater number of MyoD+ cells than medium alone (Fig. 5, A and B). The stimulation of MyoD+ cell number by testosterone and DHT was blocked by 3- to 10-fold molar excess of bicalutamide.

Effects of testosterone and DHT on adipogenesis are blocked by an AR antagonist

As expected, incubation with DHT alone resulted in significantly fewer adipocytes compared with medium alone (mean: 11 adipocytes per field in DHT *vs.* 21 in control wells, $P < 0.01$). Coincubation of cells with DHT and graded concentrations of bicalutamide resulted in a dose-dependent

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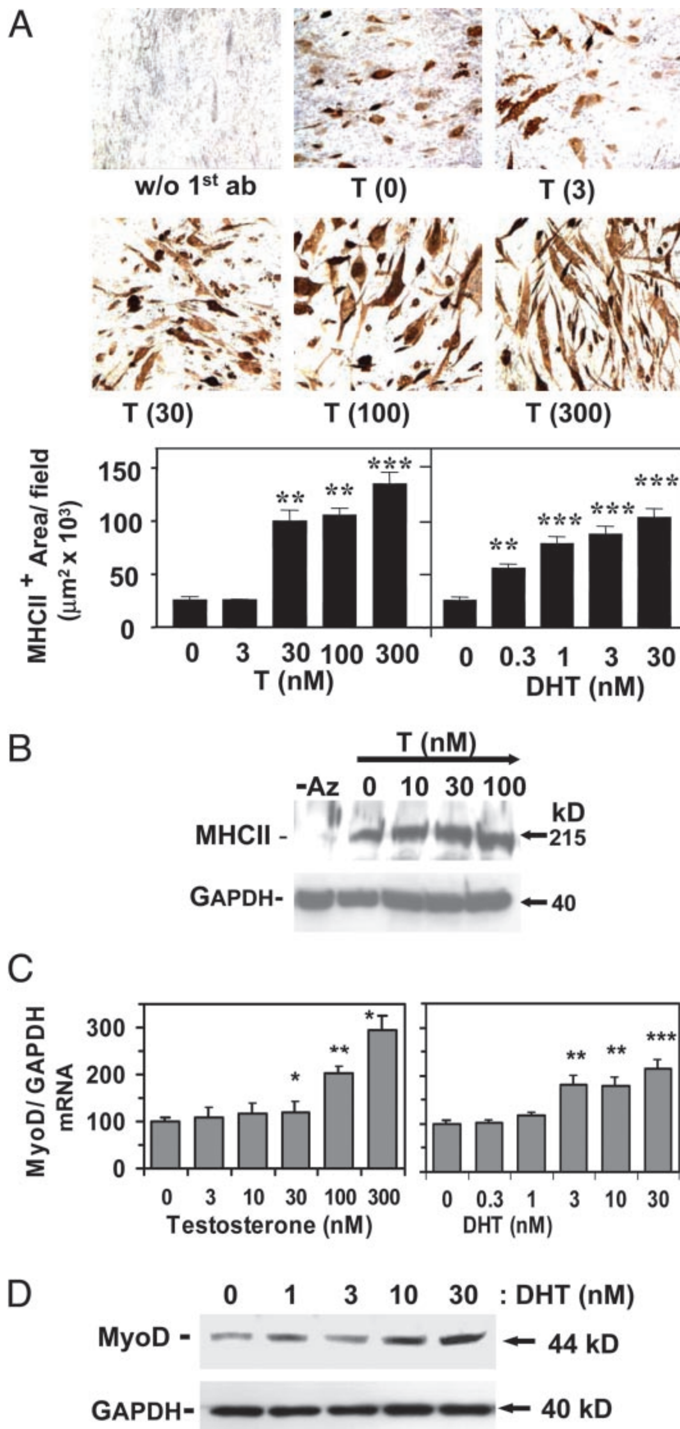

FIG. 2. Testosterone (T) and DHT stimulate myogenesis in 10T1/2 cells A, Immunocytochemical staining of MHC+ myogenic cells. The *upper panel* shows 10T1/2 cells treated for 12 d with T (0–300 nM) or DHT (0–30 nM, data not shown) and analyzed by immunocytochemistry using an anti-MHC antibody. Negative control did not include the first antibody (w/o 1st ab). Total areas of MHC+ cells per field are plotted in the *lower panel* (*P* values *vs.* control: **, $P < 0.01$; ***, $P < 0.001$). Magnification, ×100. B, Western blot analysis of MHC protein. Cell extracts were analyzed by immunoblotting using anti-MHC or anti-GAPDH antibody. The protein size (kDa) and T concentrations (nM) are shown. −Az, Cells not treated with 5-azacytidine. C, Quantitation of MyoD mRNA by real-time RT-PCR. 10T1/2 cells were treated with T (0–300 nM) or DHT (0–30 nM). MyoD and GAPDH mRNA ratios were analyzed by real-time RT-PCR (*P* values *vs.* control: *, $P < 0.05$; **, $P < 0.03$; ***, $P < 0.001$). D, Western blot analysis of MyoD protein. Cell extracts were analyzed by immunoblotting using anti-MyoD or anti-GAPDH antibody. DHT concentrations (nM) are shown.

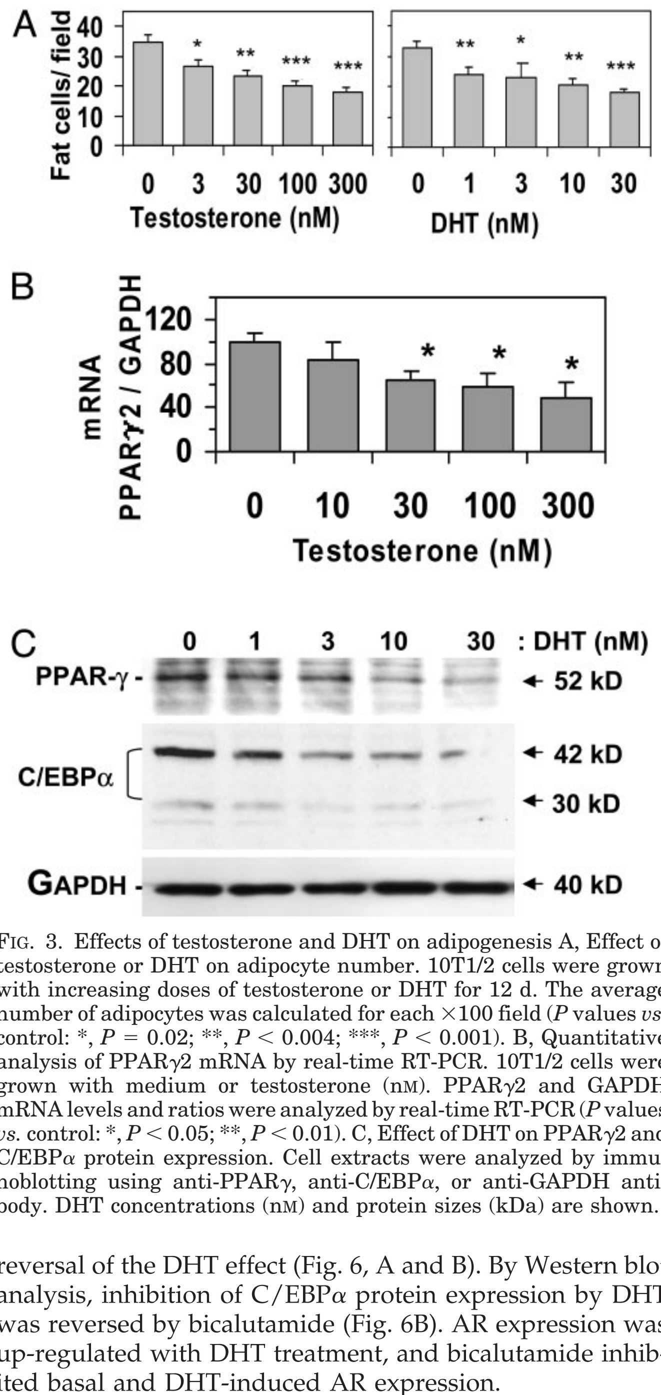

FIG. 3. Effects of testosterone and DHT on adipogenesis A, Effect of testosterone or DHT on adipocyte number. 10T1/2 cells were grown with increasing doses of testosterone or DHT for 12 d. The average number of adipocytes was calculated for each ×100 field (*P* values *vs.* control: *, $P = 0.02$; **, $P < 0.004$; ***, $P < 0.001$). B, Quantitative analysis of PPARγ2 mRNA by real-time RT-PCR. 10T1/2 cells were grown with medium or testosterone (nM). PPARγ2 and GAPDH mRNA levels and ratios were analyzed by real-time RT-PCR (*P* values *vs.* control: *, $P < 0.05$; **, $P < 0.01$). C, Effect of DHT on PPARγ2 and C/EBPα protein expression. Cell extracts were analyzed by immunoblotting using anti-PPARγ, anti-C/EBPα, or anti-GAPDH antibody. DHT concentrations (nM) and protein sizes (kDa) are shown.

reversal of the DHT effect (Fig. 6, A and B). By Western blot analysis, inhibition of C/EBPα protein expression by DHT was reversed by bicalutamide (Fig. 6B). AR expression was up-regulated with DHT treatment, and bicalutamide inhibited basal and DHT-induced AR expression.

Discussion

These data demonstrate that testosterone and DHT regulate the differentiation of C3H 10T1/2 pluripotent cells of mesenchymal origin; these androgens stimulate myogenic differentiation while inhibiting adipogenic differentiation. The effects of testosterone and DHT on pluripotent cell dif-

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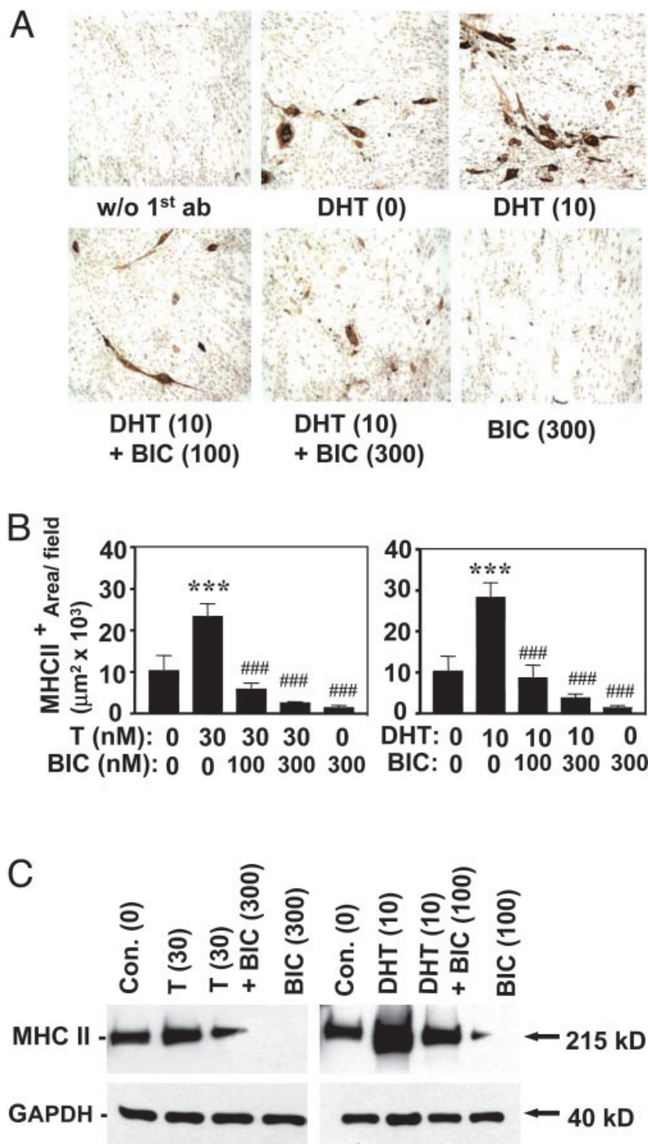

FIG. 4. A, AR antagonist, bicalutamide (BIC), blocks the effects of testosterone (T) and DHT on MHC expression in 10T1/2 cells. 10T1/2 cells were treated for 12 d with DHT (nM) or T (data not shown) with or without BIC. MHC expression was visualized by immunocytochemistry (magnification, ×100) using anti-MHC antibody. w/o 1st antibody, without first antibody. B, Effects of BIC (nM) on area of MHC+ myotubes in wells treated with T (*left*) or DHT (*right*). The total areas of the MHC+ myotubes per field are shown (*P* values *vs.* control: ***, $P < 0.001$; *P vs.* T or DHT: ###, $P < 0.001$). C, Effects of AR blockade on MHC protein by Western blotting. Cell extracts were analyzed by immunoblotting using an anti-MHC or anti-GAPDH antibody. T, DHT, and BIC concentrations (nM) are shown. Con., control.

ferentiation are mediated through an AR-dependent mechanism because they are blocked by bicalutamide, an AR antagonist. AR expression in the uncommitted 10T1/2 cells was induced by androgens, suggesting that androgens modulate pluripotent cell responsiveness by up-regulating the expression of their own receptor. The effects of testosterone and DHT on pluripotent cell differentiation are detectable at concentrations that are close to the circulating concentrations of these hormones. Our data support the hypothesis that

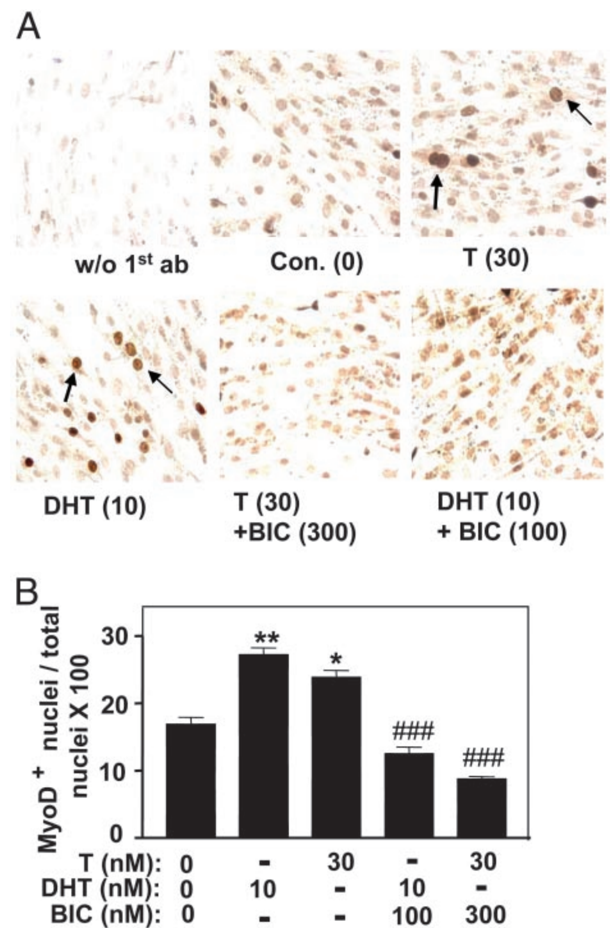

FIG. 5. AR antagonist inhibits androgen effects on MyoD expression A, Immunocytochemistry of MyoD in 10T1/2 cells. 10T1/2 cells grown in growth medium were treated for 6 d with DHT (10 nM) or testosterone (T) (30 nM) with or without bicalutamide (BIC). MyoD expression visualized by immunocytochemistry using an anti-MyoD antibody is shown from representative wells (magnification, ×40). w/o 1st antibody, Without first antibody. B, Effect of AR antagonist on the number of MyoD+ cells. MyoD+ cells were expressed as a percent of the total number of hematoxylin-stained nuclei (*P* values *vs.* control: *, $P < 0.05$; **, $P < 0.01$; *P vs.* DHT alone: ###, $P < 0.001$).

androgens regulate body composition, at least partly, by stimulating the commitment of pluripotent cells that are resident within the skeletal muscle and elsewhere into the myogenic lineage and inhibiting their progression into the adipogenic pathway.

The anabolic effects of androgens on the skeletal muscle have been controversial for over six decades, primarily due to problems of study design (1); the nature and the reasons for this controversy have been discussed in excellent reviews (1). However, recent studies (2–7) have demonstrated that replacement doses of testosterone, when given to hypogonadal men, increase fat-free mass and decrease fat mass. Administration of supraphysiological doses of testosterone to eugonadal men leads to further gains in fat-free mass, muscle size, and maximal voluntary strength (12). Testosterone effects on fat-free mass are directly correlated with the administered dose and the serum testosterone concentrations; in contrast, the changes in whole-body and regional fat mass are inversely correlated with testosterone dose and

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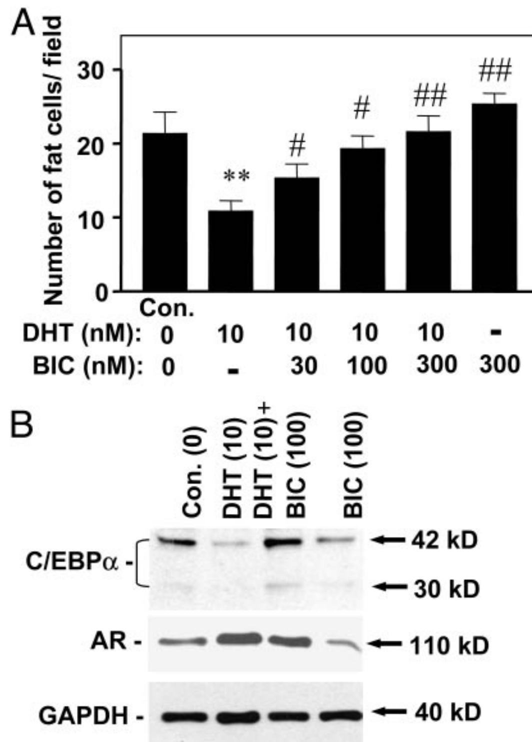

FIG. 6. A, AR antagonist blocks DHT effects on adipocyte number. 10T1/2 cells treated with DHT for 12 d were stained with oil red O. The average number of adipocytes was expressed as a percent of hematoxylin-positive nuclei (*P* values *vs.* control: **, $P < 0.01$; *P vs.* DHT alone: #, $P < 0.05$; ##, $P < 0.01$). B, AR antagonist blocks DHT effects on C/EBPα and AR protein. Protein extracts were separated on 7.5% agarose gels and detected by immunoblotting. The concentrations (nM) of DHT and bicalutamide (BIC) are shown in *parentheses*. Con. (0), Control wells treated with vehicle alone; T, testosterone.

concentrations (13). Thus, testosterone is an important regulator of body composition in men.

The mechanisms by which testosterone regulates body composition are poorly understood. Observations that testosterone administration increases nitrogen retention in castrated male mammals (16) led to the hypothesis that testosterone increases muscle mass by stimulating muscle protein synthesis. Studies in young, hypogonadal men (5) and older men with low testosterone levels (18–19) have reported gains in fractional muscle protein synthesis after testosterone administration. However, the hypothesis that muscle protein synthesis is the primary site of androgen action does not adequately explain either the increases in myonuclear and satellite cell number that accompany testosterone supplementation (15) or the reciprocal decrease in fat mass (13). Our hypothesis that testosterone affects body composition by regulating pluripotent cell differentiation provides a unifying explanation for the observed discordant changes in muscle and fat mass caused by testosterone supplementation. Increased commitment of pluripotent cells into the myogenic lineage would also be consistent with the observed increase in the number of satellite cells. However, it is possible that significant changes in muscle protein synthesis and/or degradation may accompany induction of myogenesis by testosterone administration. We cannot exclude the possibility that androgens might also affect additional steps in the differentiation of myogenically committed cells, such as the satellite cells.

The 10T1/2 cells are a recognized *in vitro* model of cell differentiation that have been used widely to define the earlier stages of myogenesis (26–28); however, no experimental model can replicate the complexity of the living human organism. Treatment with 5-azacytidine or transfection with cDNAs for MyoD or Myf5 can induce a fraction of these cells to differentiate into myogenic cells (26–28), leading to the appearance of mononuclear myoblasts (26–28). After the removal of certain key growth factors, myoblasts fuse into polynucleated myotubes that express MHC and other proteins characteristic of the differentiated muscle fibers. However, the late stages of myogenesis in which myotubes become organized into functional muscle bundles and develop innervation and vascularization are not represented in this *in vitro* model. Also, because of the murine fetal origin of 10T1/2 cells, we do not know whether the changes observed in these cells can be extrapolated to native pluripotent stem cells of adult humans. Although 10T1/2 cells mimic many differentiation features of the adult pluripotent stem cells and are a widely used model for studying the regulation of stem-cell commitment to myogenesis or adipogenesis, it would be premature to apply these data directly to human myogenesis. Further studies are needed to confirm the effects of androgens on native, adult, pluripotent stem cells.

Although there is now agreement that testosterone administration increases muscle mass in men, the effects of androgen administration on fat mass are not as well recognized. Androgens inhibit whole-body fat mass; thus, androgen deficiency in men is associated with higher fat mass compared with eugonadal controls (3), and testosterone supplementation decreases whole-body and intermuscular fat mass (6–9). De Pergola (36) has reported that testosterone inhibits differentiation of adipocyte precursor cells, suppresses lipid uptake and lipoprotein lipase activity in adipocytes, and up-regulates the number of β-adrenergic receptors. In our study, the expression of the markers of adipogenic differentiation, such as PPARγ2 and C/EBPα, was down-regulated, and the number of adipocytes was inhibited in a dose-dependent manner by both testosterone and DHT. Our data are consistent with those of James *et al.*, (37) who observed a significant decrease in the number of fat cells in the inguinal and retroperitoneal region of female rats treated with supraphysiological doses of testosterone. Taken together, these data suggest that androgens inhibit adipogenic commitment of mesenchymal pluripotent cells, resulting in the formation of fewer adipocytes. However, we do not know whether androgens have additional effects on preadipocyte differentiation because C3H 10T1/2 cells are not an optimum model of preadipocyte differentiation.

The androgen effects on both myogenic and adipogenic differentiation were blocked by bicalutamide, indicating that these effects are mediated through an AR-mediated pathway. It has been speculated that the anabolic effects of supraphysiological doses of androgens might represent an antiglucocorticoid effect; our data do not exclude this possibility. However, almost complete inhibition of the effects of both testosterone and DHT by bicalutamide is con-

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sistent with an important role of the AR pathway in mediating these effects on 10T1/2 cell differentiation.

Although both testosterone and DHT were effective in stimulating myogenesis and inhibiting adipogenesis, DHT was more potent than testosterone in this model. These data confirm previous observations that suggest that DHT is more potent than testosterone in some androgen-responsive tissues (34). Pregnenolone, a steroid precursor of testosterone, had no significant effect on the differentiation of 10T1/2 cells, indicating that these effects are specific to androgenic steroids.

Significant effects of testosterone and DHT on pluripotent cell differentiation were observed at concentrations that bracket the circulating levels of these hormones in young men. However, incubation with testosterone and DHT concentrations that were higher than the upper limit of the normal male range was associated with greater stimulation of myogenesis and greater inhibition of adipogenesis than with physiological concentrations.

Under basal conditions, 10T1/2 cells expressed AR mRNA and protein in low abundance, and testosterone and DHT up-regulated the expression of AR mRNA and protein in these cells. The up-regulation of AR by androgens may provide a mechanism for potentiation of androgen responsiveness. Ferrando *et al.* (18) have also reported an increase in AR expression in the skeletal muscle of men after androgen administration.

In this *in vitro* model, we did not find a complete concordance in the magnitude of changes in the mRNA and protein concentrations of myogenic markers and the change in the number of MHC+ myotubes. We only studied a limited number of time points, and it is possible that the time course for the optimal induction of different myogenic markers is different from that of myotube formation. Therefore, it is not surprising that the changes in steady-state concentrations of mRNA and protein concentrations do not always show complete proportionality to phenotypic changes observed at a specific time point in cell differentiation.

These data do not exclude the possibility that androgens might have additional effects on muscle protein synthesis, satellite cell replication, myoblast fusion, myogenic progression to fully differentiated fibers, and one or more steps in the adipogenic differentiation pathway. However, the reciprocal effects of androgens on myogenic and adipogenic differentiation suggest that these hormones likely act at sites that are proximal to both the myogenic and adipogenic differentiation pathways and involve mechanisms for lineage determination in mesenchymal precursor cells. The molecular mechanisms by which androgens regulate lineage commitment are unknown. Previous studies have suggested that the Wnt signaling pathway plays an important role in lineage determination in pluripotent stem cells (32, 35). Further studies are needed to determine the role of Wnt and other signaling pathways in mediating androgen action on pluripotent stem-cell differentiation.

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Address all correspondence and requests for reprints to: Shalender Bhasin, M.D., Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R. Drew University of Medicine and Science, 1731 East 120th Street, Los Angeles, California 90059. E-mail: sbhasin@ucla.edu.

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R.S., J.N.A., and W.E.T. contributed equally to this work as first authors.

N.F.G.-C. and S.B. contributed equally.

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