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Effects of Testosterone Supplementation on Skeletal Muscle Fiber Hypertrophy and Satellite Cells in Community-Dwelling Older Men

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Objective: In this study, we determined the effects of graded doses of testosterone on muscle fiber cross-sectional area (CSA) and satellite cell number and replication in older men.

Participants: Healthy men, 60–75 yr old, received a long-acting GnRH agonist to suppress endogenous testosterone production and 25, 50, 125, 300, or 600 mg testosterone enanthate im weekly for 20 wk.

Methods: Immunohistochemistry, light and confocal microscopy, and electron microscopy were used to perform fiber typing and quantitate myonuclear and satellite cell number in vastus lateralis biopsies, obtained before and after 20 wk of treatment.

Results: Testosterone administration in older men was associated with dose-dependent increases in CSA of both types I and II fibers. Satellite cell number increased dose dependently at the three highest doses (3% at baseline *vs.* 6.2, 9.2, and 13.0% at 125, 300, and 600 mg doses, $P < 0.05$). Testosterone administration was associated with an increase in the number of proliferating cell nuclear antigen+ satellite cells (1.8% at baseline *vs.* 3.9, 7.5, and 13% at 125, 300, and 600 mg doses, $P < 0.005$). The expression of activated Notch, examined only in the 300-mg group (baseline, 2.3 *vs.* 9.0% after treatment, $P < 0.005$), increased in satellite cells after testosterone treatment. The expression of myogenin (baseline, 6.2 *vs.* 20.7% after treatment, $P < 0.005$), examined only in the 300-mg group, increased significantly in muscle fiber nuclei after testosterone treatment, but Numb expression did not change.

Conclusions: Older men respond to graded doses of testosterone with a dose-dependent increase in muscle fiber CSA and satellite cell number. Testosterone-induced skeletal muscle hypertrophy in older men is associated with increased satellite cell replication and activation. **(*J Clin Endocrinol Metab* 91: 3024–3033, 2006)**

AGING IN HUMANS is associated with a progressive decrease in skeletal muscle mass and strength (1–3), resulting in an increased risk of mobility disorders, falls, and fractures (3–5). The age-related loss of muscle mass reflects largely a preferential loss of type II skeletal muscle fibers (6, 7).

Testosterone is being evaluated as an anabolic therapy for age-related physical dysfunction (8). Systematic reviews of literature (8) have concluded that testosterone supplementation increases skeletal muscle mass in hypogonadal men (9–11), men with chronic illness (12, 13), and healthy, older men (14–19). However, the mechanisms by which testosterone increases skeletal muscle mass are poorly understood and were the focus of this investigation.

It has been suggested that skeletal muscle of older animals is less capable of adaptive response to injury or anabolic stimuli (20, 21), although similar data are not available in older humans. Therefore, we investigated the changes in muscle fiber cross-sectional area (CSA) in healthy older men in response to administration of graded doses of testosterone. Because muscle satellite cells play an important role in mediating the hypertrophic response to anabolic stimulus and muscle injury, we determined the effects of testosterone administration on satellite cell number in older men in muscle biopsies obtained during the course of a testosterone dose-response study (14). We compared these data with those obtained previously in healthy, young men (22).

Changes in satellite cell number could occur due to either changes in rates of satellite cell replication or satellite cell apoptosis. We used immunohistochemical staining for proliferating cell nuclear antigen (PCNA) as a marker for satellite cell entry into the cell cycle. Based on data in porcine satellite cells (23), we hypothesized that testosterone administration would be associated with an increase in PCNA+ satellite cells, consistent with an increase in satellite cell entry into the cell cycle.

Notch-signaling pathway is essential for satellite cell activation during embryogenesis as well as during adult muscle regeneration (20, 21, 24, 25). Skeletal muscle regeneration in older mice has been reported to be impaired in comparison with younger mice due to impaired capacity of satellite cells to proliferate and form myoblasts (20, 24). Conboy *et al.* (24) have suggested that decreased regenerative potential of skeletal muscle in older rats is related to diminished Notch activation (24). Numb is an inhibitor of Notch signaling that interacts with the intracellular portion of Notch and antagonizes its activity by preventing nuclear translocation (24, 25). It has been proposed that interplay between Notch and

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Abbreviations: CSA, Cross-sectional area; LC-MS/MS, liquid chromatography tandem mass spectrometry; MHC, myosin heavy chain; MRF, myogenic regulatory factor; PCNA, proliferating cell nuclear antigen.

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its antagonist Numb determines postnatal myogenesis (25). Because activation of Notch-1 promotes the proliferation of myogenic precursor cells (25), we considered the possibility that testosterone increases satellite cell replication by activating Notch signaling pathway. Accordingly, we studied the changes in activated Notch expression in response to testosterone administration in older men. In addition, we evaluated the expression of myogenin protein as a marker of satellite cell activation and myogenic differentiation.

Subjects and Methods

Experimental design

The Institutional Review Boards of Charles R. Drew University of Medicine and Science and Los Angeles Biomedical Research Institute (Torrance, CA) approved the protocol. All participants provided written, informed consent.

The study design, inclusion, and exclusion criteria have been published previously (14). Briefly, 60 healthy older men aged 60–75 yr were treated with monthly injections of GnRH agonist (Lupron depot, 7.5 mg) to suppress endogenous testosterone production and weekly im injections of 25, 50, 125, 300, or 600 mg testosterone enanthate for 20 wk (Table 1).

The volume of the thigh muscle was measured by magnetic resonance imaging (Signa Horizons LX scanner, General Electric Medical Systems, Milwaukee, WI) before and after 20 wk of treatment as described by us previously (26). In brief, magnetic resonance imaging scans were obtained from the entire thigh, with the first slice taken at the distal border of the lateral femoral condyle. A total of 17 slices, each of 10-mm thickness, were taken. The thigh muscle volume was then calculated by integrating the transverse slices by use of commercially available software (General Electric Volume Analysis software, AW version 3.1).

We obtained muscle biopsies from the vastus lateralis muscle group before and after 20 wk of treatment in 41 participants (Fig. 1). Of these, data on 36 subjects, who had both baseline and posttreatment biopsies of satisfactory quality, are reported here. As described previously, energy and protein intake were standardized (14). Subjects were prescribed a diet standardized for energy (150 $kJ/kg^{-1}\cdot d^{-1}$) and protein (1.3 $g/kg^{-1}\cdot d^{-1}$). Dietary instructions were reinforced monthly, and compliance was verified using 3-d food records. The subjects were asked not to participate in sports events, resistance exercise training, or moderate to heavy endurance exercise during the study. These instructions were reinforced every 2 wk.

Hormone assays

Serum total testosterone levels were measured by a previously reported RIA that has been validated against liquid chromatography tandem mass spectrometry (LC-MS/MS) (27, 28). Free testosterone was separated by an equilibrium dialysis procedure and measured by RIA (27, 28). The RIA and LC-MS/MS methods (15) were compared by analyzing samples prepared in charcoal-stripped serum to which known amounts of testosterone had been added. These measurements demonstrated a correlation of 0.99 between the RIA and LC-MS/MS measurements (28). The intra- and interassay coefficients of variation were 8.2 and 13.2%, respectively.

Muscle biopsy and tissue fixation

Percutaneous needle muscle biopsies were obtained from the midregion of the vastus lateralis muscle before treatment and after 20 wk of testosterone administration. Each muscle biopsy was divided into two portions; one portion was fixed in 10% formalin (26) and the other in 5% glutaraldehyde in 0.05 M cacodylate buffer (22). Formalin-fixed tissues were processed and embedded in paraffin and used for light microscopic, morphometric, and immunohistochemical studies. Glutaraldehyde-fixed tissues were used for electron microscopy. The electron micrographs were used to determine satellite cell proportion.

Additional muscle tissue, snap frozen in liquid nitrogen immediately after biopsy, was available for Western blotting for a limited number of subjects.

Muscle fiber typing

Muscle fibers were identified as type I and type II fibers by immunohistochemical staining using a mouse monoclonal antibody (Sigma, St. Louis, MO) specific for the heavy chain of fast myosin (type II fibers). This antibody recognizes all subtypes of type II myosin heavy chains (MHC) but does not cross-react with type I MHC (26). The slides were counterstained with hematoxylin. The fibers expressing mixed myosin heavy chains were not counted separately but counted as type I or type II fiber, depending on the prevalent expression of myosin heavy chain (more than 50%).

Histomorphometry

An RG-3 grid (square lattice with 121 intersections) in a Leitz Wetzler (Wetzler, Germany) (HM-LUX) microscope was used to determine CSAs of type I and type II muscle fibers (29). A minimum of 100 muscle fibers of each type were analyzed in each biopsy specimen. The fields were randomly selected, and all muscle fibers encompassed in these fields were evaluated.

Myonuclear number

Myonuclei were stained using hematoxylin and were directly counted in 100 muscle fibers encompassed in randomly selected fields.

Identification and determination of satellite cell number

Glutaraldehyde-fixed tissues were postfixed in 1% osmium tetra oxide and processed for epoxy embedding. Embedded tissue blocks were sectioned with an LKB ultramichrotome. Thin sections showing pale gold interference were stained with uranyl acetate and lead citrate and examined with a electron microscope (Hitachi, Tokyo, Japan). The satellite cells were identified on the basis of their characteristic location and ultrastructural morphology (22, 30) by using the following criteria (see Fig. 4C): 1) the satellite cells are mononucleated cells with independent cytoplasm; and 2) the satellite cells are situated inside the basal lamina of the muscle fiber but are separated from the fiber by a sarcolemma.

All satellite cells were counted from 300 electron micrographs obtained from each subject before and after treatment, as described previously (22). The number of satellite cells was expressed as a percent of total myonuclear number in the muscle fiber (see a typical myonucleus shown in Fig. 4D).

TABLE 1. Baseline characteristics of the participants

	Testosterone dose (mg)				
	25	50	125	300	600
Age (yr)	65 ± 1	63 ± 2	67 ± 2	68 ± 2	67 ± 3
Height (cm)	183 ± 10	180 ± 2	175 ± 4	178 ± 2	172 ± 4
Weight (kg)	78 ± 1	80 ± 3	78 ± 4	86 ± 6	85 ± 9
Serum testosterone (ng/dl)	397 ± 58	339 ± 27	401 ± 28	355 ± 49	333 ± 55
Thigh muscle volume (cm^3)	1417 ± 59	1474 ± 92	1376 ± 66	1515 ± 122	1694 ± 94

The baseline characteristics of the 36 men in whom evaluable baseline and posttreatment biopsies were available are shown. The data are mean ± SEM. There were no significant differences for any of the baseline variables among the five treatment groups. To convert testosterone levels from nanograms per deciliter to nanomoles per liter, multiply the values in nanograms per deciliter by 0.0347.

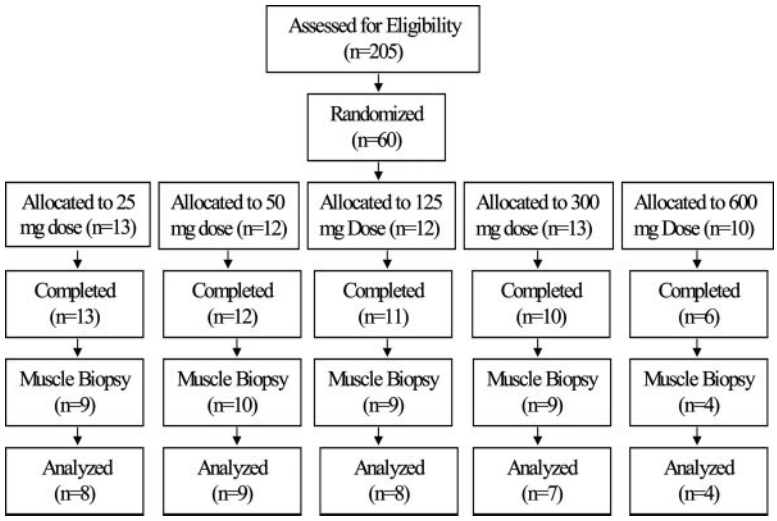


FIG. 1. The flow of subjects through the study. Forty-one men underwent muscle biopsies; evaluable baseline and posttreatment muscle biopsies were available in 36 men.

Identification of PCNA+ satellite cells and nuclei

We used double immunolabeling by using anti-syndecan-4 antibody to mark basal lamina of the muscle fiber, the sarcolemma separating the satellite cells, and anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) antibody to mark proliferating satellite cells. The slides were counterstained with Harris hematoxylin to identify all the nonproliferating myonuclei and satellite cells. The number of muscle fibers with PCNA+ nuclei was expressed as a percent of total number of muscle fibers (number of muscle fibers with PCNA+ nuclei divided by the total number of muscle fibers × 100). In addition, we computed the number of PCNA+ muscle fiber nuclei as a percent of total number of nuclei inside the basal lamina (number of PCNA+ nuclei divided by the total number of muscle fiber nuclei), as described by Mozdziak *et al.* (31) and Mesires and Doumit (32).

Myogenin+ satellite cells

In men treated with the 300-mg testosterone dose, we used antimyogenin antibody (Sc 576; Santa Cruz) to identify satellite cells that had become committed to myogenic differentiation. We used hematoxylin to stain all the muscle fiber nuclei. The number of myogenin+ nuclei was expressed as a percent of total number of nuclei in the muscle fiber.

Activated Notch+ satellite cells

In men treated with the 300-mg testosterone dose, we used antibodies against activated Notch (5545; Santa Cruz Inc.) to evaluate the effects of testosterone administration on Notch activation in muscle fibers. The cells expressing activated Notch were counted and expressed as percent of total muscle fiber nuclei. Confocal microscopy was used to colocalize activated Notch and PCNA.

For all immunohistochemical staining, sections that were processed concurrently without the addition of primary antibody served as negative controls.

Western blotting

The specificity of the antibodies used for immunohistochemistry was confirmed by Western blot analysis. Western blots were performed with lysates of muscle biopsies from the 300-mg group using antibodies against δ-1 (SC 7423, Santa Cruz), Notch 2 (SC 5545, Santa Cruz), myogenin (SC576), and Numb-like antigen (MGC 3139, Protein Tech Group, Chicago, IL) and the ECL Western blot analysis system (Amersham, Chicago, IL).

Statistical analyses

All data are presented as mean ± SE for each of the five treatment groups. We used one-way ANOVA to compare between-group differences. If overall ANOVA revealed significant differences, *post hoc* comparisons were performed using Tukey's test or Dunn's multiple comparison method. Paired *t* tests were used to evaluate the differences between pre- and posttreatment values in each group. Pearson correlation coefficients were calculated to establish relationships between variables. Wherever applicable, the data obtained from older men (muscle fiber CSA, proportions of type I and II muscle fibers) were compared with previously reported data in healthy young men.

For all statistical analyses, a 0.05 level of significance was used. Sigma-Stat program (SPSS, Chicago, IL) was used for all statistical analyses.

Results

Baseline characteristics of the participants

The details of study design have been published previously (14). Briefly, of the 60 older men who were randomized, 52 completed the study. Forty-one participants underwent muscle biopsies; satisfactory baseline and posttreatment muscle biopsies were available in 36 participants: eight in the 25-mg testosterone enanthate group, nine in the 50-mg group, eight in the 125-mg group, seven in the 300-mg group, and four in the 600-mg group (Table 1 and Fig. 1).

The five treatment groups did not differ significantly in their baseline characteristics (Table 1). Serum-free and total testosterone concentration at baseline were significantly lower (Table 2) in older men in comparison with young men reported previously (22, 33). The combined administration of GnRH agonist and graded doses of testosterone enanthate resulted in dose-dependent changes in serum total and free testosterone concentrations in this sample of 36 men as it did in the entire sample of 52 men (14). The mean CSA of type I muscle fibers in vastus lateralis did not differ significantly between the young and older men at baseline, but the mean CSA of type II muscle fibers in older men was significantly smaller than in young men at baseline (Table 2). The number of satellite cells, expressed as a percent of muscle fiber nuclei, was not significantly different between the young and older men (Table 2).

Muscle fiber composition

The relative proportion of types I and II muscle fibers at baseline was not significantly different among five treatment groups (Fig. 2). Administration of GnRH agonist plus tes-

TABLE 2. Comparison of baseline characteristics of older men reported in this manuscript and young men whose data have been reported previously (22)

Variable	Young men (n = 39)	Older men (n = 36)	*P* value
Total testosterone (ng/dl)	605 ± 204	368 ± 19	<0.001
Free testosterone (pg/ml)	63.8 ± 3.6	36.6 ± 2.4	<0.001
Type I muscle fiber CSA (μm^2)	3422 ± 117	3570 ± 60	NS
Type II fiber CSA (μm^2)	3710 ± 134	3157 ± 55	<0.001
Satellite cell (%)	2.3 + 0.2	3.0 + 0.7	NS

The data generated from older men in this manuscript were compared with historical data in young men that have been reported previously (22, 29). Data are mean ± SEM. To convert total testosterone levels in nanograms per deciliter to SI units (nanomoles per liter), multiply total testosterone values in nanograms per deciliter by 0.0347. To convert free testosterone levels from picograms per milliliter to SI units (picomoles per liter), multiply the value in picograms per milliliter by 3.47. Satellite cell number is expressed as a percent of myonuclear number.

tosterone enanthate did not significantly change the proportion of types I and II fibers. However, older men had a smaller proportion of type II muscle fibers than young men. Also, in some subjects, MHC II isoforms were found to be mixed with type I MHC isoform to give muscle fibers a mixed appearance (Fig. 3D). In some older men, as many as one third of the muscle fibers were determined to be of mixed fibers when compared with younger men, whereas less than 5% of the fibers were of mixed composition.

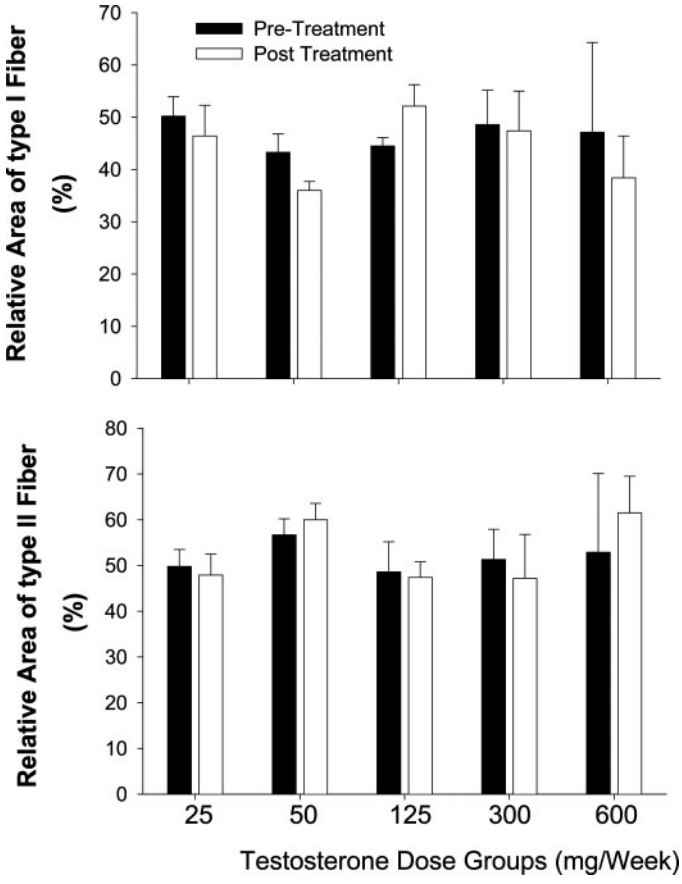


FIG. 2. Effects of testosterone treatment on relative proportion (expressed as percent) of types I and II skeletal muscle fibers in older men (mean ± SEM). The *upper panel* represents relative proportion of type I fiber, and the *lower panel* represents the area of type II fiber. The relative proportion of muscle fiber type did not vary significantly with testosterone treatment at any dose.

Muscle fiber CSA

Testosterone treatment was associated with dose- and concentration-dependent increases in CSA of both types I and II fibers in older men (Fig. 3, A and D). Groups receiving 300 mg (baseline *vs.* wk 20 type I fiber CSA, 3588 ± 289 *vs.* 4221 ± 161 μm^2; type II fiber CSA, 3172 ± 245 *vs.* 3673 ± 326 μm^2) and 600 mg testosterone enanthate (baseline *vs.* wk 20, type I fiber CSA, 3532 + 84 *vs.* 5039 + 134; type II fiber CSA, 3180 + 81 *vs.* 4338 + 306 μm^2) experienced significant increases in type I ($P < 0.001$) and type II ($P < 0.005$) muscle fiber CSA, and these changes in muscle fiber CSA in the 300- and 600-mg dose groups were significantly greater than those in the 25-mg dose group (Fig. 3A). The changes in fiber CSA of both type I and II fibers were correlated with changes in total (type 1: r = 0.575, $P < 0.001$; type II: r = 0.476, $P < 0.005$) (Fig. 3B) and free (type 1: r = 0.534, $P < 0.001$; type II: r = 0.401, $P = 0.05$) testosterone levels (Fig. 3C).

Myonuclear and satellite cell numbers

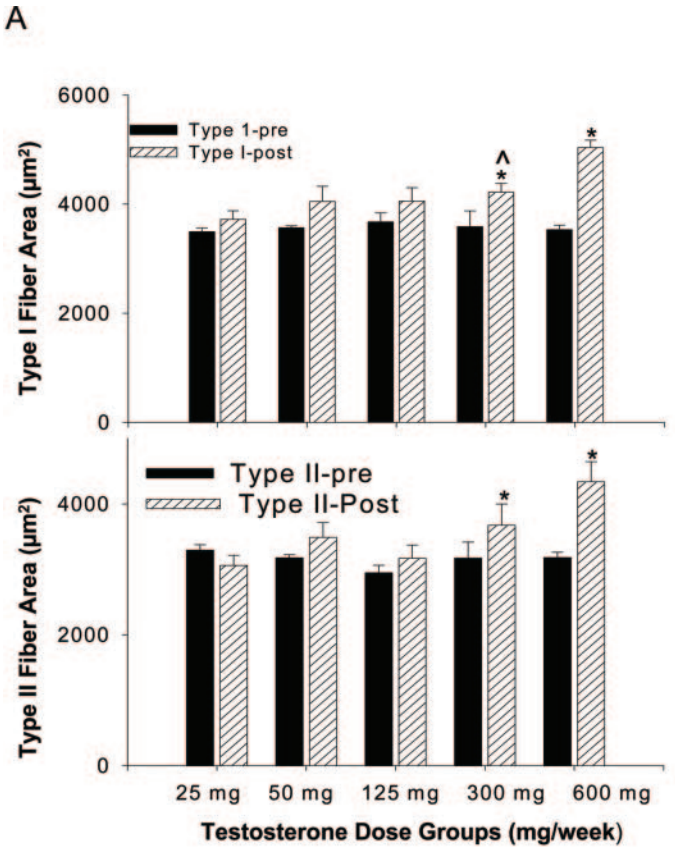
The average myonuclear number was not significantly different in the five treatment groups at baseline. Testosterone administration was associated with a dose-dependent increase in myonuclear number ($P < 0.005$) (Fig. 4A, *upper panel*). Significant increases in myonuclear number were observed in the 125-, 300-, and 600-mg dose groups, and the increase in myonuclear number in the 600-mg dose group was significantly greater than that in the 25- and 50-mg groups.

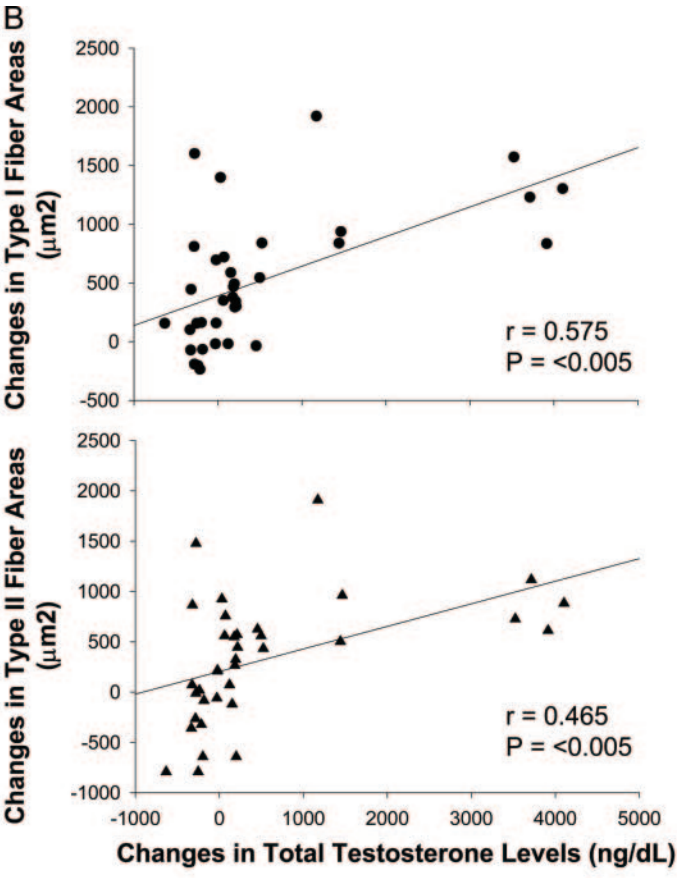
At baseline, the satellite cell number varied from 2.3 to 3.5% of the myonuclear number in older men. In response to testosterone administration, the satellite cell number increased dose dependently ($P < 0.05$); significant increases above baseline were noted in the 600-mg dose group (Fig. 4A, *lower panel*). The changes in satellite cell number were correlated with the changes in serum testosterone levels ($P = 0.535$, r = 0.04) but incidentally not with free testosterone levels ($P = 0.479$, r = 0.08) (data not shown). The increases in satellite cell numbers in older men were comparable with those reported previously in young men.

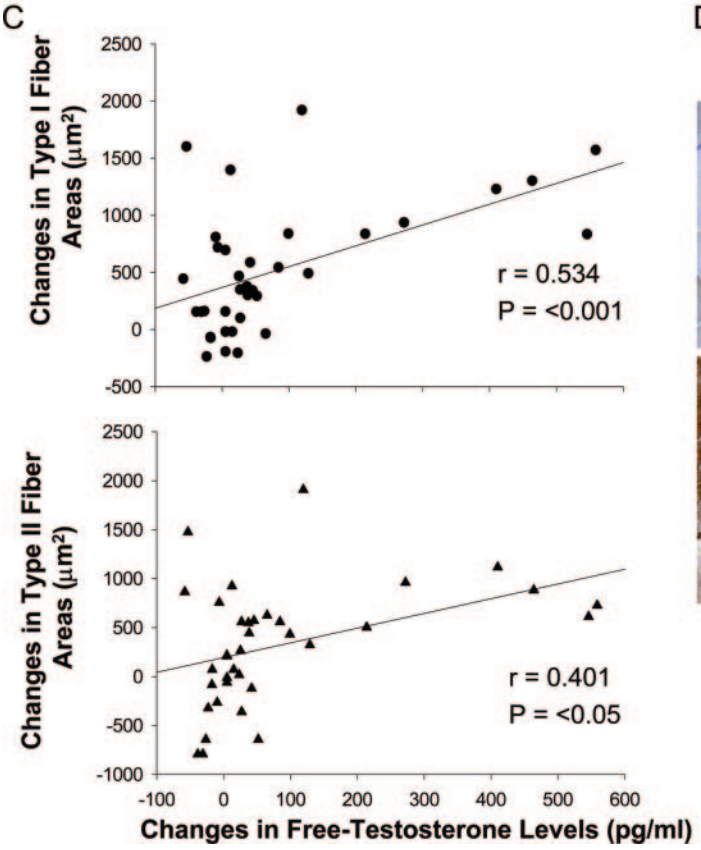
As shown in Fig. 4B, the changes in myonuclear number were correlated with the changes in serum total (r = 0.542, $P = 0.0009$) and free testosterone levels (r = 0.413, $P = 0.015$).

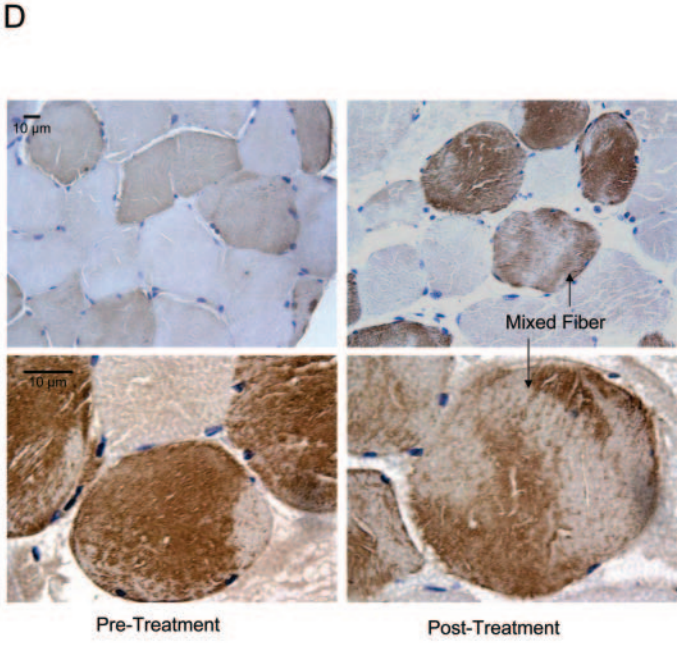
PCNA, activated Notch, and myogenin expression

Confocal microscopy revealed colocalization of active Notch and PCNA in satellite cells (Fig. 5A). However, not all the PCNA+ nuclei showed activated Notch expression (data not shown). As shown in Fig. 5B, the number of muscle fibers containing PCNA+ nuclei (ANOVA $P < 0.05$) and the percent of PCNA+ nuclei in skeletal muscle fibers (ANOVA, $P < 0.005$) were significantly greater after testosterone treatment than at baseline. We next evaluated the expression of activated Notch and myogenin proteins in men treated with the 300-mg weekly dose of testosterone enanthate because









this group had demonstrated a large increase in satellite cell number and sufficient numbers of pre- and posttreatment biopsies were available. The number of nuclei staining positive for activated Notch was higher after testosterone treatment than at baseline (9.0% after testosterone treatment *vs.* 2.3% of nuclei at baseline, $P < 0.05$, Fig. 5C). Immunoreactive myogenin expression was higher in satellite cell nuclei after treatment with testosterone enanthate than at baseline (20.7% myogenin+ satellite cell nuclei after testosterone treatment *vs.* 6.2% of myogenin+ nuclei at baseline, $P < 0.05$; Fig. 5D). However, no significant difference was observed in Numb expression after testosterone treatment (data not shown).

Discussion

Our data demonstrate that testosterone administration in community-dwelling, older men is associated with dose-related hypertrophy of both type I and II skeletal muscle fibers, although the relative proportion of type I, type II, and mixed muscle fibers remains unchanged with testosterone administration. We are also intrigued by the observation that, compared with the young men, in whom less than 5% of the fibers were of mixed composition, in some older men as many as one third of the muscle fibers were of mixed types. Whereas the significance of this finding remains unknown, we interpret this change as a refection of change in skeletal muscle cytoarchitecture associated with aging. The number of myonuclei and satellite cells increased dose dependently with testosterone administration. The number of PCNA+ muscle fiber nuclei and PCNA+ muscle fibers increased in response to testosterone administration as well, suggesting that testosterone promotes the entry of myogenic precursor cells into the cell cycle. Additionally, expression of activated Notch, an important regulator of satellite cell replication, and myogenin, a marker of satellite cell activation, was increased with testosterone administration. These data are consistent with the hypothesis that testosterone administration induces skeletal muscle hypertrophy by promoting satellite cell replication and activation resulting in an increased number of myogenically committed satellite cells (23, 34–36).

Human studies of this type, based on the immunohistochemical and histomorphometric evaluation of muscle biopsies, have several inherent limitations, small sample size in particular. In fact, in the 600-mg group, only four men underwent biopsies and are the subject for the muscle morphometry. We also obtained muscle biopsies at a single time point, 20 wk after testosterone treatment. Therefore, transient changes in muscle gene expression would not be detected. In addition, vastus lateralis may not be representative of all skeletal muscle groups. Our data suggest an association of testosterone treatment with satellite cell activation and differentiation markers but cannot establish a cause-and-effect relationship or the time course of this response. Furthermore, our data do not exclude the involvement or even predominance of other signaling pathways, such as muscle protein synthetic or degradation pathways (37), in mediating testosterone-induced muscle fiber hypertrophy. Therefore, the inference drawn from these data should be guarded and confirmed in nonhuman experimental models.

Our data also demonstrate that skeletal muscle fibers of older men are capable of undergoing substantial hypertrophy in response to testosterone administration. Although the proportion of type II muscle fibers is lower in older men than young men (26), the magnitude of type I and type II muscle fiber hypertrophy observed in older men after testosterone administration was not significantly different from that reported previously in young men (26). Also, the hypertrophic responses of both type I and II muscle fibers were correlated with the administered testosterone dose. It is possible that small differences in the responsiveness of young and older men might have been missed due to type II error. It is also pertinent to note here that testosterone supplementation in healthy older men augments IGF-I production (38), which is known to influence muscle growth (39, 40). Thus, we cannot rule out the possibility that IGF-I may have also contributed, at least in part, to such testosterone-induced muscle hypertrophy. It is possible that the observed effects of testosterone on muscle may result from its antiglucocorticoid effect. In animal models, glucocorticoid antagonizes the effects of testosterone; conversely, testosterone administration can prevent glucocorticoid-induced muscle atrophy (reviewed in Ref. 41). Also, androgen administration up-regulates androgen receptor expression in the skeletal muscle (42); it is possible that testosterone may increase the responsiveness of the skeletal muscle to androgen action by up-regulating its own receptors. These possibilities clearly require further investigations.

The formation of skeletal muscle is a well-orchestrated multistep process controlled by the myogenic regulatory factors (MRFs). The MRF family consists of four members including, Myf5, MyoD, myogenin, and MRF4, all of which bind to sequence-specific DNA elements present in the promoter of muscle gene (reviewed in Ref. 43). The MRFs have the unique property of converting nonmuscle cells to muscle lineage. Satellite cells play a critical role in skeletal muscle adaptation to injury or hypertrophic stimuli (43–48). When activated, satellite cells undergo proliferation, differentiation to myoblast formation by MRFs, such as myogenin and MyoD, and self-renewal by asymmetric cell division (48–50). Inhibition of satellite cell proliferation by γ-irradiation prevents muscle growth that follows muscle atrophy due to hind

FIG. 3. A, Effects of testosterone administration on types I and II muscle fiber CSA in healthy, older men treated with a long-acting GnRH agonist and graded doses of testosterone enanthate for 20 wk. Data are mean ± SEM. *, Significantly different from baseline; ^, significantly different from 25-mg dose. B, Correlation between change in serum total testosterone concentrations during treatment and change in CSA of type I (*upper panel*) and type II (*lower panel*) muscle fibers. C, Correlation in changes in serum-free testosterone concentrations during treatment and changes in muscle fiber CSAs of type I (*upper panel*) and type II fibers (*lower panel*). D, Cross-sections of muscle biopsies before and after testosterone treatment in one man treated with GnRH agonist and 300 mg testosterone enanthate weekly. The *left panel* shows cross sections of muscle fibers at baseline at ×400 (*upper panel*) and at ×1000 magnification (*lower panel*). The *right panel* shows cross sections of muscle fibers after testosterone treatment at ×400 (*upper panel*) and at ×1000 magnification (*lower panel*). The fibers are immunostained with an antibody against MHC specific for type II fibers and are stained *brown*. *Light blue* fibers are type I fibers, stained with hematoxylin. In older men the fibers showing mixed expression of MHC of types I and II fibers are common as can be seen in this picture.

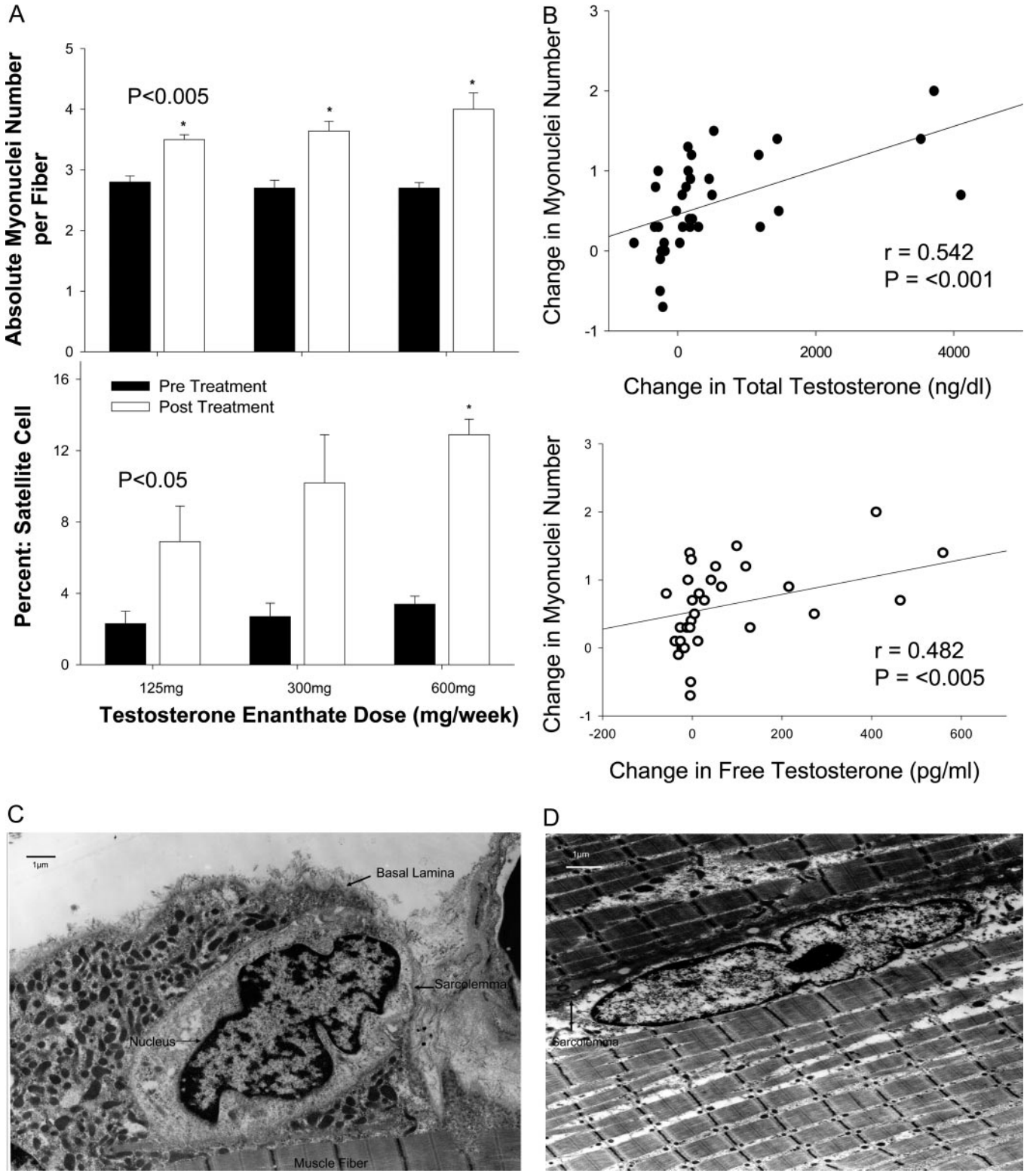


FIG. 4. A, Effect of testosterone treatment on myonuclear number per muscle fiber (*upper panel*, mean ± SEM) and percent of muscle satellite cell (*lower panel*, mean ± SEM) in relation to total muscle fiber nuclei. *, Significantly different from baseline. B, Correlation between the changes in myonuclear number and the changes in serum-free testosterone concentrations during treatment. C, Electron micrograph of a typical satellite cell. The micrograph illustrates the two essential identifying features of a satellite cell: 1) it resides inside the basal lamina of the fiber and separated from the fiber by sarcolemma; and 2) satellite cell has its own nucleus surrounded by cytoplasm. *Scale bar*, 1 μm. D, Electron micrograph of a typical myonucleus. Note that unlike satellite cell, a myonucleus is located inside the sarcolemma and does not contain an independent cytoplasm. *Scale bar*, 1 μm.

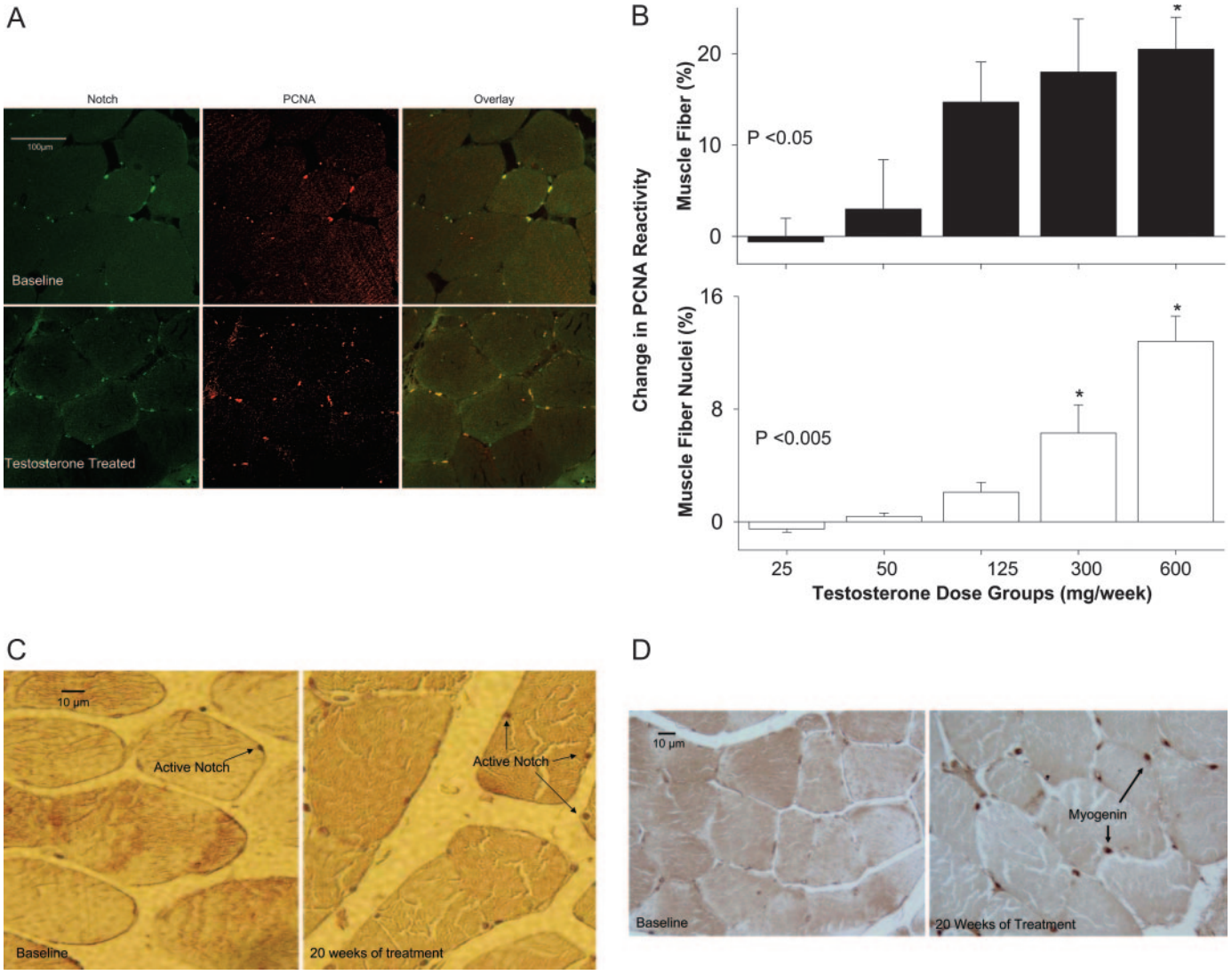


FIG. 5. A, Immunofluorescence staining for Notch (*green*, *left panels*) and PCNA (*red*, *middle panels*) in vastus lateralis cross sections by confocal microscopy. The overlaid images, showing in *yellow* the nuclei that stained for both PCNA and Notch, are displayed in the *right panels*. *Upper panels* show baseline cross sections and *lower panels* cross sections obtained after 20 wk of treatment with GnRH agonist and 300-mg testosterone enanthate weekly in a representative subject. B, Image analysis of PCNA immunostaining, expressed as a percent of either muscle fibers showing PCNA immunostaining (*upper panel*) or nuclei that were positive for PCNA immunoreactivity (*lower panel*). Data are mean ± SEM. *, Significantly different from 25- and 50-mg dose levels. C, Immunohistochemical staining for active Notch in a representative subject from 300-mg treatment group at baseline (*left panel*) and after 20 wk of treatment (*right panel*). D, Myogenic expression in a representative subject before (*left panel*) and after 20 wk of treatment with GnRH agonist and 300-mg testosterone enanthate weekly (*right panel*). Ten-micrometer *bar* shows the relative size of objects.

limb suspension (44). Muscle remodeling and repair after injury often involve satellite cell replication and recruitment of new stem cells into the myogenic cell lineage (34–36). Similarly, hypertrophy of levator ani muscle in female rats induced with exogenous testosterone administration is associated with increased satellite cell entry into the cell cycle (51). Taken together, these animal data suggest that an increase in satellite cell number may be an important antecedent of an increase in myonuclear number and muscle fiber hypertrophy. Our data constitute the first demonstration that even in older men, testosterone administration induces substantial increase in satellite cell activation and number. These data also confirm previous findings generated in the levator ani muscle of young rodents (23, 35, 36, 51) and vastus lateralis of healthy, young men (22).

The mechanisms by which testosterone increases satellite cell number are not known, but increases in satellite cell number could result from an increase in satellite cell replication, inhibition of satellite cell apoptosis, or increased differentiation of mesenchymal stem cells into the myogenic lineage. Testosterone administration induces a rapid proliferative response in muscle satellite cells of female rats (36) and neonatal pigs (23), suggesting a role for testosterone in satellite cell regulation. Our previous studies suggest that testosterone promotes the differentiation of mesenchymal multipotent cells into myogenic lineage (52) in a multipotent

embryonic cell line. This finding needs further confirmation in native populations of mesenchymal stem cells. The effect of testosterone on satellite cell apoptosis could not be evaluated in this study due to limited availability of tissue.

Notch, a transmembrane receptor that regulates the specification of a variety of cell types, plays an important role in somatic mesoderm development (24, 25, 53, 54). The intracellular domain of Notch translocates to the nucleus and activates a number of transcription factors, thus regulating cell fate. Our data show that testosterone administration is associated with increased expression of activated Notch and PCNA, consistent with increased satellite cell replication. The mechanisms by which testosterone regulates Notch expression are unknown and should be investigated.

Myogenin expression increased with testosterone administration as well. We do not know whether increased myogenin expression reflects a direct effect of testosterone on myogenin transcription or whether this was an indirect effect, resulting from an increased number of myogenically committed cells. Because biopsies were obtained only at a single time point during treatment, the time course of these events could not be determined.

Although very substantial skeletal muscle remodeling is achievable with testosterone administration, even in older men, the magnitude of these anabolic effects is limited by the dose-limiting adverse effects of testosterone in older men. An understanding of the mechanistic pathways that mediate testosterone-induced muscle fiber hypertrophy may unveil novel targets for the development of anabolic therapies.

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References

1. **Roy TA, Blackman MR, Harman SM, Tobin JD, Schrager M, Metter EJ** 2002 Interrelationships of serum testosterone and free testosterone index with FFM and strength in aging men. Am J Physiol Endocrinol Metab 283:E284–E294
2. **Baumgartner RN, Koehler KM, Gallagher D, Romero L, Heymsfield SB, Ross RR, Garry PJ, Lindeman RD** 1998 Epidemiology of sarcopenia among the elderly in New Mexico. Am J Epidemiol 147:755–763
3. **Melton 3rd LJ, Khosla S, Crowson CS, O'Connor MK, O'Fallon WM, Riggs BL** 2000 Epidemiology of sarcopenia. J Am Geriatr Soc 48:625–630
4. **Baumgartner RN** 2000 Body composition in healthy aging. Ann NY Acad Sci 904:437–448
5. **Roubenoff R, Hughes VA** 2000 Sarcopenia: current concepts. J Gerontol A Biol Sci Med Sci 55:M716–M724
6. **Lexell J, Downham D, Sjostrom M** 1984 Distribution of different fibre types in human skeletal muscles. A statistical and computational study of the fibre type arrangement in m. vastus lateralis of young, healthy males. J Neurol Sci 65:353–365
7. **Lexell J, Downham D, Sjostrom M** 1986 Distribution of different fibre types in human skeletal muscles. Fibre type arrangement in m. vastus lateralis from three groups of healthy men between 15 and 83 years. J Neurol Sci 72:211–222
8. **Bhasin S, Calof O, Storer TW, Lee M, Mazer N, Jasuja R, Montori V, Gao W, Dalton J** 2006 Drug insight: testosterone and selective androgen receptor modulators as anabolic therapies for physical dysfunction in chronic illness and aging. Nature Clin Pract Endocrinol Metab 2:146–159
9. **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
10. **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
11. **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
12. **Bhasin S, Storer TW, Javanbakht M, Berman N, Yarasheski KE, Phillips J, Dike M, Sinha-Hikim I, Shen R, Hays RD, Beall G** 2000 Testosterone replacement and resistance exercise in HIV-infected men with weight loss and low testosterone levels. JAMA 283:763–770
13. **Grinspoon S, Corcoran C, Askari H, Schoenfeld D, Wolf L, Burrows B, Walsh M, Hayden D, Parlman K, Anderson E, Basgoz N, Klibanski A** 1998 Effects of androgen administration in men with the AIDS wasting syndrome. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 129:18–26
14. **Bhasin S, Woodhouse L, Casaburi R, Singh AB, Mac RP, Lee M, Yarasheski KE, Sinha-Hikim I, Dzekov C, Dzekov J, Magliano L, Storer TW** 2005 Older men are as responsive as young men to the anabolic effects of graded doses of testosterone on the skeletal muscle. J Clin Endocrinol Metab 90:678–688
15. **Snyder PJ, Peachey H, Hannoush P, Berlin JA, Loh L, Lenrow DA, Holmes JH, Dlewati A, Santanna J, Rosen CJ, Strom BL** 1999 Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. J Clin Endocrinol Metab 84:2647–2653
16. **Page ST, Amory JK, Bowman FD, Anawalt BD, Matsumoto AM, Bremner WJ, Tenover JL** 2005 Exogenous testosterone (T) alone or with finasteride increases physical performance, grip strength, and lean body mass in older men with low serum T. J Clin Endocrinol Metab 90:1502–1510
17. **Kenny AM, Prestwood KM, Gruman CA, Marcello KM, Raisz LG** 2001 Effects of transdermal testosterone on bone and muscle in older men with low bioavailable testosterone levels. J Gerontol A Biol Sci Med Sci 56:M266–M272
18. **Morley JE, Perry 3rd HM, Kaiser FE, Kraenzle D, Jensen J, Houston K, Mattammal M, Perry Jr HM** 1993 Effects of testosterone replacement therapy in old hypogonadal males: a preliminary study. J Am Geriatr Soc 41:149–152
19. **Ferrando AA, Sheffield-Moore M, Yeckel CW, Gilkison C, Jiang J, Achacosa A, Lieberman SA, Tipton K, Wolfe RR, Urban RJ** 2002 Testosterone administration to older men improves muscle function: molecular and physiological mechanisms. Am J Physiol Endocrinol Metab 282:E601–E607
20. **Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA** 2005 Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433:760–764
21. **Conboy IM, Rando TA** 2005 Aging, stem cells and tissue regeneration: lessons from muscle. Cell Cycle 4:407–410
22. **Sinha-Hikim I, Roth SM, Lee MI, Bhasin S** 2003 Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. Am J Physiol Endocrinol Metab 285:E197–E205
23. **Mulvaney DR, Marple DN, Merkel RA** 1988 Proliferation of skeletal muscle satellite cells after castration and administration of testosterone propionate. Proc Soc Exp Biol Med 188:40–45
24. **Conboy IM, Conboy MJ, Smythe GM, Rando TA** 2003 Notch-mediated restoration of regenerative potential to aged muscle. Science 302:1575–1577
25. **Conboy IM, Rando TA** 2002 The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3:397–409
26. **Sinha-Hikim I, Artaza J, Woodhouse L, Gonzalez-Cadavid N, Singh AB, Lee MI, Storer TW, Casaburi R, Shen R, Bhasin S** 2002 Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. Am J Physiol Endocrinol Metab 283:E154–E164
27. **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
28. **Choi HH, Gray PB, Storer TW, Calof OM, Woodhouse L, Singh AB, Padero C, Mac RP, Sinha-Hikim I, Shen R, Dzekov J, Dzekov C, Kushnir MM, Rockwood AL, Meikle AW, Lee ML, Hays RD, Bhasin S** 2005 Effects of testosterone replacement in human immunodeficiency virus-infected women with weight loss. J Clin Endocrinol Metab 90:1531–1541
29. **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 83:1312–1318
30. **Roth SM, Martel GF, Ivey FM, Lemmer JT, Metter EJ, Hurley BF, Rogers MA**

2000 Skeletal muscle satellite cell populations in healthy young and older men and women. Anat Rec 260:351–358
31. **Mozdziak PE, Pulvermacher PM, Schultz E** 2001 Muscle regeneration during hindlimb unloading results in a reduction in muscle size after reloading. J Appl Physiol 91:183–190
32. **Mesires NT, Doumit ME** 2002 Satellite cell proliferation and differentiation during postnatal growth of porcine skeletal muscle. Am J Physiol Cell Physiol 282:C899–C906
33. **Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW** 2001 Testosterone dose-response relationships in healthy young men. Am J Physiol Endocrinol Metab 281:E1172–E1181
34. **Doumit ME, Cook DR, Merkel RA** 1996 Testosterone up-regulates androgen receptors and decreases differentiation of porcine myogenic satellite cells *in vitro*. Endocrinology 137:1385–1394
35. **Joubert Y, Tobin C** 1995 Testosterone treatment results in quiescent satellite cells being activated and recruited into cell cycle in rat levator ani muscle. Dev Biol 169:286–294
36. **Joubert Y, Tobin C** 1989 Satellite cell proliferation and increase in the number of myonuclei induced by testosterone in the levator ani muscle of the adult female rat. Dev Biol 131:550–557
37. **Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, Wolfe RR** 1998 Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. Am J Physiol 275:E864–E871
38. **Veldhuis JD, Keenan DM, Mielke K, Miles JM, Bowers CY** 2005 Testosterone supplementation in healthy older men drives GH and IGF-1 secretion without potentiating peptidyl secretagogue efficacy. Eur J Endocrinol 153:577–586
39. **Ground MD** 2002 Reasons for the degeneration of ageing skeletal muscle: a central role of IGF-1 signaling. Biogerontology 3:19–24
40. **Barbieri M, Ferrucci L, Emila R** 2003 Chronic inflammation and the effect of IGF-1 on muscle strength and power in older persons. Am J Physiol Metab 284:E481–E487
41. **Bhasin S, Taylor W, Singh R, Artaza J, Sinha-Hikim I, Jasujs R, Choi H, Gonzalez-Cadavid NF** 2003 The mechanism of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action. J Gerontol Med Sci 12:1103–1110
42. **Mauras N, Hayes V, Welch S** 1998 Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength and adiposity. J Clin Endocrinol Metab 83:1886–1893
43. **Lluis F, Perdiguero E, Nebreda AR, Munoz-Canoves P** 2006 Regulation of skeletal muscle gene expression by p38 MAP kinases. Trends Cell Biol 16: 36–44
44. **Adams GR, Caiozzo VJ, Haddad F, Baldwin KM** 2002 Cellular and molecular responses to increased skeletal muscle loading after irradiation. Am J Physiol Cell Physiol 283:C1182–C1195
45. **Kadi F, Eriksson A, Holmner S, Thornell LE** 1999 Effects of anabolic steroids on the muscle cells of strength-trained athletes. Med Sci Sports Exerc 31:1528–1534
46. **Kadi F, Thornell LE** 2000 Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. Histochem Cell Biol 113:99–103
47. **Mitchell PO, Pavlath GK** 2004 Skeletal muscle atrophy leads to loss and dysfunction of muscle precursor cells. Am J Physiol Cell Physiol 287:C1753–C1762
48. **Schultz E, McCormick KM** 1994 Skeletal muscle satellite cells. Rev Physiol Biochem Pharmacol 123:213–257
49. **Darr KC, Schultz E** 1987 Exercise-induced satellite cell activation in growing and mature skeletal muscle. J Appl Physiol 63:1816–1821
50. **Schultz E** 1989 Satellite cell behavior during skeletal muscle growth and regeneration. Med Sci Sports Exerc 21:S181–S186
51. **Nnodim JO** 2001 Testosterone mediates satellite cell activation in denervated rat levator ani muscle. Anat Rec 263:19–24
52. **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
53. **Dahlqvist C, Blokzijl A, Chapman G, Falk A, Dannaeus K, Ibanez CF, Lendahl U** 2003 Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development 130:6089–6099
54. **Miller JB, Emerson Jr CP** 2003 Does the road to muscle rejuvenation go through Notch? Sci Aging Knowledge Environ 2003:pe34

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