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Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy

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Sinha-Hikim, Indrani, Jorge Artaza, Linda Woodhouse, Nestor Gonzalez-Cadavid, Atam B. Singh, Martin I. Lee, Thomas W. Storer, Richard Casaburi, Ruoquing Shen, and Shalender Bhasin. Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. *Am J Physiol Endocrinol Metab* 283: E154–E164, 2002. First published March 12, 2002; 10.1152/ajpendo.00502.2001.—Administration of replacement doses of testosterone to healthy hypogonadal men and supraphysiological doses to eugonadal men increases muscle size. To determine whether testosterone-induced increase in muscle size is due to muscle fiber hypertrophy, 61 healthy men, 18–35 yr of age, received monthly injections of a long-acting gonadotropin-releasing hormone (GnRH) agonist to suppress endogenous testosterone secretion and weekly injections of 25, 50, 125, 300, or 600 mg testosterone enanthate (TE) for 20 wk. Thigh muscle volume was measured by magnetic resonance imaging (MRI) scan, and muscle biopsies were obtained from vastus lateralis muscle in 39 men before and after 20 wk of combined treatment with GnRH agonist and testosterone. Administration of GnRH agonist plus TE resulted in mean nadir testosterone concentrations of 234, 289, 695, 1,344, and 2,435 ng/dl at the 25-, 50-, 125-, 300-, and 600-mg doses, respectively. Graded doses of testosterone administration were associated with testosterone dose and concentration-dependent increase in muscle volume measured by MRI (changes in vastus lateralis volume, −4, +7, +15, +32, and +48 ml at 25-, 50-, 125-, 300-, and 600-mg doses, respectively). Changes in cross-sectional areas of both type I and II fibers were dependent on testosterone dose and significantly correlated with total ($r = 0.35$, and 0.44, $P < 0.0001$ for type I and II fibers, respectively) and free ($r = 0.34$ and 0.35, $P < 0.005$) testosterone concentrations during treatment. The men receiving 300 and 600 mg of TE weekly experienced significant increases from baseline in areas of type I (baseline vs. 20 wk, 3,176 ± 186 vs. 4,201 ± 252 μm^2, $P < 0.05$ at 300-mg dose, and 3,347 ± 253 vs. 4,984 ± 374 μm^2, $P = 0.006$ at 600-mg dose) muscle fibers; the men in the 600-mg group also had significant increments in cross-sectional area of type II (4,060 ± 401 vs. 5,526 ± 544 μm^2, $P = 0.03$) fibers. The relative proportions of type I and type II fibers did not change significantly after treatment in any group. The myonuclear number per fiber increased significantly in men receiving the 300- and 600-mg doses of TE and was significantly correlated with testosterone concentration and muscle fiber cross-sectional area. In conclusion, the increases in muscle volume in healthy eugonadal men treated with graded doses of testosterone are associated with concentration-dependent increases in cross-sectional areas of both type I and type II muscle fibers and myonuclear number. We conclude that the testosterone induced increase in muscle volume is due to muscle fiber hypertrophy.

androgen; muscle hypertrophy; satellite cells; mechanism of action

TESTOSTERONE ADMINISTRATION in replacement doses to hypogonadal men (9, 12, 26, 43, 46) and in supraphysiological doses to healthy eugonadal men (8, 16, 18, 49) is associated with significant increases in fat-free mass and muscle size. Similarly, testosterone replacement in men infected with the human immunodeficiency virus, who are experiencing weight loss and have low testosterone levels, induces significant gains in lean body mass with concomitant increases in muscle volume (7, 10, 19). We have recently demonstrated that changes in circulating testosterone concentrations, induced by combined administration of gonadotropin-releasing hormone (GnRH) agonist and graded doses of testosterone, are associated with testosterone dose- and concentration-dependent changes in fat-free mass and muscle size (11). However, the mechanisms by which testosterone increases muscle mass are not well understood. We do not know whether testosterone-induced increase in muscle mass is due to muscle fiber hypertrophy, muscle cell hyperplasia, or both. The effects of testosterone administration on muscle fiber size and composition in humans are unknown, and the data from experimental animals are both limited and somewhat contradictory. For instance, in a study by

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Tucek et al. (45), testosterone treatment of castrated rats led to a 15% increase in the weight of the levator ani muscle; this increase in muscle mass was accompanied by an increase in the size of muscle fibers. Tobin and Joubert (44) reported a significant sex difference in both the number of fibers and their average cross-sectional areas for levator ani muscle and speculated that the higher fiber number and size in the male rats might be related to the higher testosterone levels. However, a recent study (17) did not find any differences in the cross-sectional areas of fast- and slow-twitch fibers between older men and women with vastly different androgen concentrations.

Therefore, the overall objective of the present study was to determine whether testosterone-induced increase in muscle size is due to increase in muscle fiber area or number or both. We treated healthy young men with a long-acting GnRH agonist to suppress their endogenous testosterone production and one of five different doses of testosterone enanthate to create different circulating testosterone concentrations. Muscle biopsies were obtained before and after 20 wk of combined treatment with GnRH agonist and testosterone to determine the morphometric changes in muscle fibers of vastus lateralis muscle. We assessed whether the morphometric changes in the vastus lateralis muscle fibers were correlated with testosterone dose and circulating testosterone concentrations and whether these changes were fiber type specific.

MATERIALS AND METHODS

Subjects. This was a double-blind, randomized study. The details of the study design have been previously published (11). Briefly, the participants were 61 healthy eugonadal men, 18–35 yr of age. Each participant provided informed consent approved by the institutional review boards of Charles R. Drew University and Harbor-UCLA Research and Education Institute. Participants were randomly assigned to one of five experimental groups to receive monthly injections of a long-acting GnRH agonist to suppress endogenous testosterone production and weekly injections of 25, 50, 125, 300, or 600 mg of testosterone enanthate for 20 wk. The lowest testosterone dose regimen, 25 mg/wk, was selected because previous studies had shown that this weekly dose of testosterone can maintain sexual function in GnRH antagonist-treated men (35). The selection of the 600-mg weekly regimen was based on the consideration that this was the highest dose of testosterone enanthate that had been shown to be safe and effective in increasing muscle size and strength in healthy eugonadal men in short-term clinical trials (8). The staff of the General Clinical Research Center administered testosterone and GnRH agonist injections to ensure compliance.

Of the 61 men, who were randomized, 54 completed all phases of the study (11). Thus body composition and muscle volume data were available on 54 men. Of these, 39 men underwent pre- and posttreatment muscle biopsies: nine in the 25-mg group, seven in the 50-mg group, eight in the 125-mg group, nine in the 300-mg group, and six in the 600-mg group.

Nutritional intake and exercise stimulus were controlled at the outset of the study, as previously described (11). Two weeks before the initiation of the study, subjects were prescribed a diet standardized for energy intake at 150 $kJ \cdot kg^{-1} \cdot day^{-1}$ and protein intake at 1.3 $g \cdot kg^{-1} \cdot day^{-1}$. These instructions were reinforced every 4 wk during a meeting with the dietitian, in which subject's actual nutrient intake was verified by analysis of 3-day food records.

Hormone assays. Serum total testosterone was measured by a previously reported radioimmunoassay (7–11). Free testosterone was separated by an equilibrium dialysis procedure and measured by radioimmunoassay (42). The sensitivity of total testosterone assay was 0.6 ng/dl; intra-assay coefficient of variation was 8.2%, and interassay coefficient of variation was 13.2%. For free testosterone assay, the sensitivity was 0.22 pg/ml, and intra- and interassay coefficients of variation were 4.2 and 12.3%.

Quadriceps muscle volume. The volume of the quadriceps muscle group was measured by magnetic resonance imaging (MRI; Signa Horizons LX Scanner, General Electric Medical Systems, Milwaukee, WI) before and after 20 wk of treatment. The software used for data acquisition was Signa MR Scan Assistant, version 8.3. Subjects entered the body coil feet first and lay in a supine position with the heels in a neutral position. MRI scans were obtained for 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, was 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, Milwaukee, WI). Accuracy of the volume analysis software was determined by scanning and analyzing a phantom cylinder of known dimensions. Duplicate manual tracings were drawn around the outermost edge of the entire thigh (total thigh), the skeletal musculature (to subtract out subcutaneous fat), and the femur (to subtract out femoral bone areas). The quadriceps musculature was measured by manually tracing around the vastus lateralis, vastus medialis, vastus intermedius, and rectus femoris muscles. The middle transverse slice with the largest diameter was used to measure cross-sectional areas of the vastus lateralis. A single individual, who was unaware of the group assignments, performed all of the analyses.

Muscle biopsy and tissue fixation. Percutaneous needle muscle biopsies were obtained from the midbelly of the vastus lateralis muscle in the quadriceps muscle group before and within 5 days after the final testosterone enanthate injection. Each muscle biopsy was divided into two portions; one portion was fixed in 10% formalin and the other in Bouin's fixative. Both formalin- and Bouin's-fixed tissues were oriented on their longitudinal axis and embedded in paraffin and used for light microscopic, morphometric, and immunohistochemical studies. In some individuals, in whom additional muscle tissue was available, the tissue was snap-frozen in liquid nitrogen in a solution of RNAase for subsequent extraction of RNA.

Muscle fiber typing. Muscle fibers in the vastus lateralis were identified as type I and type II fibers by immunohistochemical staining using a mouse monoclonal antibody (clone MY 32, Sigma, St Louis, MO) specific for the heavy chain of fast myosin. This antibody recognizes all subtypes of type II myosin heavy chain (MHC) but does not cross-react with type I MHC (3, 4, 22). Both the formalin- and Bouin's-fixed 6-μm tissue sections were immunostained using the diaminobenzidine tetrahydrochloride-based detection method (Vectastain-Elite ABC kit, Vector Labs). The slides were counterstained with Harris hematoxylin. Tissue sections that were

incubated with mouse IgG instead of the primary antibody served as negative controls.

In 19 men in whom sufficient biopsy material was available, we measured the relative mRNA concentrations of human MHC isotypes I, IIa, IIb, and IIx by reverse transcription and polymerase chain reaction (RT-PCR). The concentration of RNA, isolated by TRIzol, was estimated by spectrophotometry and verified by nondenaturing agarose gel electrophoresis, ethidium bromide staining, and densitometry of the 28S and 18S bands. MHC mRNA isotypes were determined by modification of a published procedure (48) for rat MHC isotypes. Briefly, 0.6–1 μg of total RNA was reverse transcribed with poly(dT) primer, and aliquots of this reaction were submitted to PCR utilizing a forward primer within a region identical for the four human MHC subtypes (AAGG CCAAGAAGGCCATCAC) and reverse primers that were specific for MHC I (TCCAGCTCATTCTTCAGCTC), MHC IIa (TGCCTGAAGTTTATCTACCA), MHC IIb (GGTTTGCAATTTGTCCACCA), or MHC IIx (CTTGCAGCTTGTCCACCAGG). A fifth reaction was conducted for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a "housekeeping" gene. PCR conditions for each primer set were optimized utilizing the thermal Robocycler (Stratagene, La Jolla, CA), and the final reaction conditions were standardized at 92°C for 5 min, followed by 25 cycles at 92°C for 1 min, 58°C for 1 min, and 72°C for 1 min and an extension at 72°C for 10 min. The expected amplified DNA fragment sizes were: MHC I, 221 bp, MHC IIa, 360 bp, MHC IIb, 360 bp, MHC IIx, 358 bp, and GAPDH, 1,100 bp. The intensity of the PCR product was determined by densitometry and corrected by the amount of 28S RNA or the intensity of the GAPDH band.

Because our data were derived by using the RT-PCR technique, we considered the possibility that the observed expression might be due to amplification of a closely related mRNA species (perhaps another MHC isoform). To exclude this possibility, we sequenced the product of RT-PCR for IIb mRNA. The sequence was identical to the one reported in the literature and in GenBank for the human MHC type IIb. These data provide unequivocal evidence that MHC isoform IIb mRNA is expressed in vastus lateralis.

Morphometry. The immunohistochemically stained slides were used for morphometric evaluation using an RG-3 grid (square lattice with 121 intersections) in a Leitz Wetzler (HM-LUX) microscope. An observer who was unaware of the group assignments evaluated all the slides. Fiber typing was carried out by directly counting type I and type II muscle fibers in a known area ($9{,}216 \times 15\ \mu m^2$) of tissue sections. We counted ≥500 muscle fibers in each biopsy specimen. The relative abundance of type I and II fibers was expressed as a percentage of the total fiber number.

The cross-sectional area of the muscle fibers (A) was determined by point counting using the equation $A = p \cdot \mu^2$, where p is number of points per fiber profile and μ is the distance between two neighboring points, the magnification used being taken into account (3, 41). A minimum of 150 type II fibers and 100 type I fibers was analyzed in each biopsy specimen. The fields were randomly selected to measure the fiber area, and all of the fibers encompassed in those fields were evaluated.

Fiber number was determined by counting each of the fiber types per unit muscle area by use of slides that had been immunostained for MHC (2). We examined a minimum of 10 fields ($60{,}025\text{-}\mu m^2$ area) in each biopsy. We also counted the number of myonuclei in 20 randomly selected muscle fibers of each type in each sample.

Counting of blood capillaries. Sections from Bouin's-fixed tissues were stained for alkaline phosphatase activity to identify all of the capillaries surrounding the muscle fibers. The capillaries were counted at a total magnification of ×400 by use of a square frame in the eyepiece of the microscope, and expressing the amount as capillary density per unit area [unit area = 600 μm^2 (14, 40)]. The fiber-to-capillary ratio was obtained by counting all of the capillaries and fibers in five artifact-free unit areas in each section.

Statistical analyses. All data are presented as means ± SE for each of the five treatment groups. We used one-way ANOVA to compare between-group differences in the change from baseline in each outcome measure. The main outcome measures were change from baseline in muscle fiber number, relative proportion of type I and type II fibers, cross-sectional areas of type I and type II fibers, number of myonuclei per muscle fiber, capillary density, total testosterone, free testosterone, and quadriceps volume. If overall ANOVA revealed significant differences, paired *t*-tests were used to examine the differences between pre- and posttreatment values in each group. Pearson product-moment correlation coefficients were computed, using linear regression, to evaluate the relationships between serum total and free testosterone concentrations and changes in key outcome measures.

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

Of the 61 men who enrolled in the study, 54 completed the treatment (11). Thirty-nine of the men who completed the study underwent baseline and posttreatment muscle biopsies: nine in the 25-mg group, seven in the 50-mg group, eight in the 125-mg group, nine in the 300-mg group, and six in the 600-mg group. The five treatment groups did not differ significantly in their baseline characteristics (Tables 1–3).

A detailed description of the hormonal changes in all 61 treated men has been previously published (11). In the 39 men who underwent muscle biopsies and are the subject of this report, serum total and free testosterone concentrations were not significantly different among the five treatment groups at baseline (Table 2). Combined administra-

Table 1. *Baseline characteristics of the participants*

	Testosterone Dose, mg				
	25	50	125	300	600
Age, yr	27 ± 2	30 ± 2	29 ± 1	29 ± 1	26 ± 2
Height, cm	173.5 ± 1.2	175.8 ± 2.	175.8 ± 2.6	175.2 ± 2.1	173.8 ± 2.3
Weight, kg	66.9 ± 2.6	76.3 ± 3.1	78.0 ± 3.6	78.0 ± 3.6	66.9 ± 2.6

Data are means ± SE. There were no significant differences among the groups for any of these variables at baseline.

Table 2. *Serum total and free testosterone concentrations at baseline and during week 16*

	Dose, mg				
	25	50	125	300	600
Serum total testosterone concentrations, ng/dl (overall ANOVA, P < 0.001)					
Baseline	605 ± 50	562 ± 79	620 ± 70	620 ± 72	613 ± 66
Week 16	234 ± 60	289 ± 67	695 ± 83	1,344 ± 98	2,435 ± 489
Change from baseline	−371 ± 97	−264 ± 67	75 ± 83	724 ± 124	1,822 ± 492
n	9	6	7	9	6
Baseline vs. *week 16*	0.005	0.007	0.400	<0.001	0.01
Serum free testosterone concentrations, pg/ml (overall ANOVA, P < 0.001)					
Baseline	62 ± 22	60 ± 25	55 ± 24	72 ± 22	69 ± 27
Week 16	27 ± 22	33 ± 25	58 ± 24	137 ± 22	341 ± 27
n	9	6	7	9	6
Week 16 vs. baseline	0.008	0.01	0.75	0.004	0.04

Data are means ± SE; *n* = number of subjects. Testosterone concentrations were measured during *week 16*, 7 days after the previous testosterone injection, and therefore represent nadir values.

tion of GnRH agonist and graded doses of testosterone enanthate resulted in dose-dependent changes in serum total and free testosterone concentrations. Thus this treatment regimen was effective in creating and maintaining graded levels of serum total and free testosterone concentrations, providing validity to our experimental model.

Mean quadriceps and vastus lateralis muscle volumes, measured by MRI, were not significantly different among the five groups at baseline (Table 3). Combined administration of GnRH agonist and testosterone resulted in dose-dependent changes from baseline in quadriceps and vastus lateralis muscle volumes (Table 3). Administration of 300 ($P < 0.005$) and 600 mg ($P = 0.002$) of testosterone enanthate weekly was associated with significant increases in quadriceps volume.

Muscle fiber cross-sectional area. The average cross-sectional areas of type I and II muscle fibers were not significantly different among the five treatment groups at baseline (Table 3). Testosterone treatment was associated with dose- and concentration-dependent increases in the cross-sectional area of both type I and type II fibers (Fig. 1*C*). The groups receiving 300 and 600 mg of testosterone enanthate

Table 3. *Quadriceps and vastus lateralis muscle volumes and cross-sectional areas of type I and type II muscle fibers*

	Dose, mg				
	25	50	125	300	600
Quadriceps muscle volume, ml (overall ANOVA, P < 0.001)					
Baseline	441 ± 36	494 ± 39	527 ± 41	491 ± 27	520 ± 21
Week 20	431 ± 33	504 ± 43	561 ± 44	439 ± 24	595 ± 32
Change	−10 ± 11	10 ± 6	24 ± 6	49 ± 8	75 ± 13
n	9	6	7	9	6
Week 20 vs. baseline	0.419	0.163	0.007	<0.001	0.002
Vastus lateralis muscle volume, ml (overall, ANOVA, P < 0.001)					
Baseline	289 ± 24	321 ± 25	349 ± 30	301 ± 15	339 ± 14
Week 20	285 ± 23	328 ± 28	365 ± 29	333 ± 13	387 ± 21
Change	−4 ± 7	7 ± 4	15 ± 4	32 ± 5	48 ± 8
n	9	6	7	9	6
Week 20 vs. baseline	0.615	0.156	0.007	<0.001	0.002
Cross-sectional area of type I muscle fibers, μm² (overall ANOVA, P < 0.001)					
Baseline	3,337 ± 256	3,198 ± 172	3,484 ± 283	3,176 ± 186	3,347 ± 253
Week 20	3,665 ± 479	3,833 ± 253	3,674 ± 220	4,201 ± 252	4,984 ± 374
Change	328 ± 326	634 ± 208	189 ± 134	1,025 ± 164	1,637 ± 359
n	9	7	8	9	6
Week 20 vs. baseline	NS	0.02	NS	<0.001	0.006
Cross-sectional area of type II muscle fibers, μm² (overall ANOVA, P = 0.002)					
Baseline	3,973 ± 393	3,601 ± 191	3,707 ± 289	3,801 ± 254	4,060 ± 401
Week 20	3,989 ± 454	4,455 ± 238	4,011 ± 281	4,521 ± 210	5,526 ± 544
n	9	7	8	9	6
Change	16 ± 217	853 ± 247	304 ± 162	720 ± 371	1,466 ± 400
Week 20 vs. baseline	NS	NS	NS	NS	0.03

Data are means ± SE. NS, not significant.

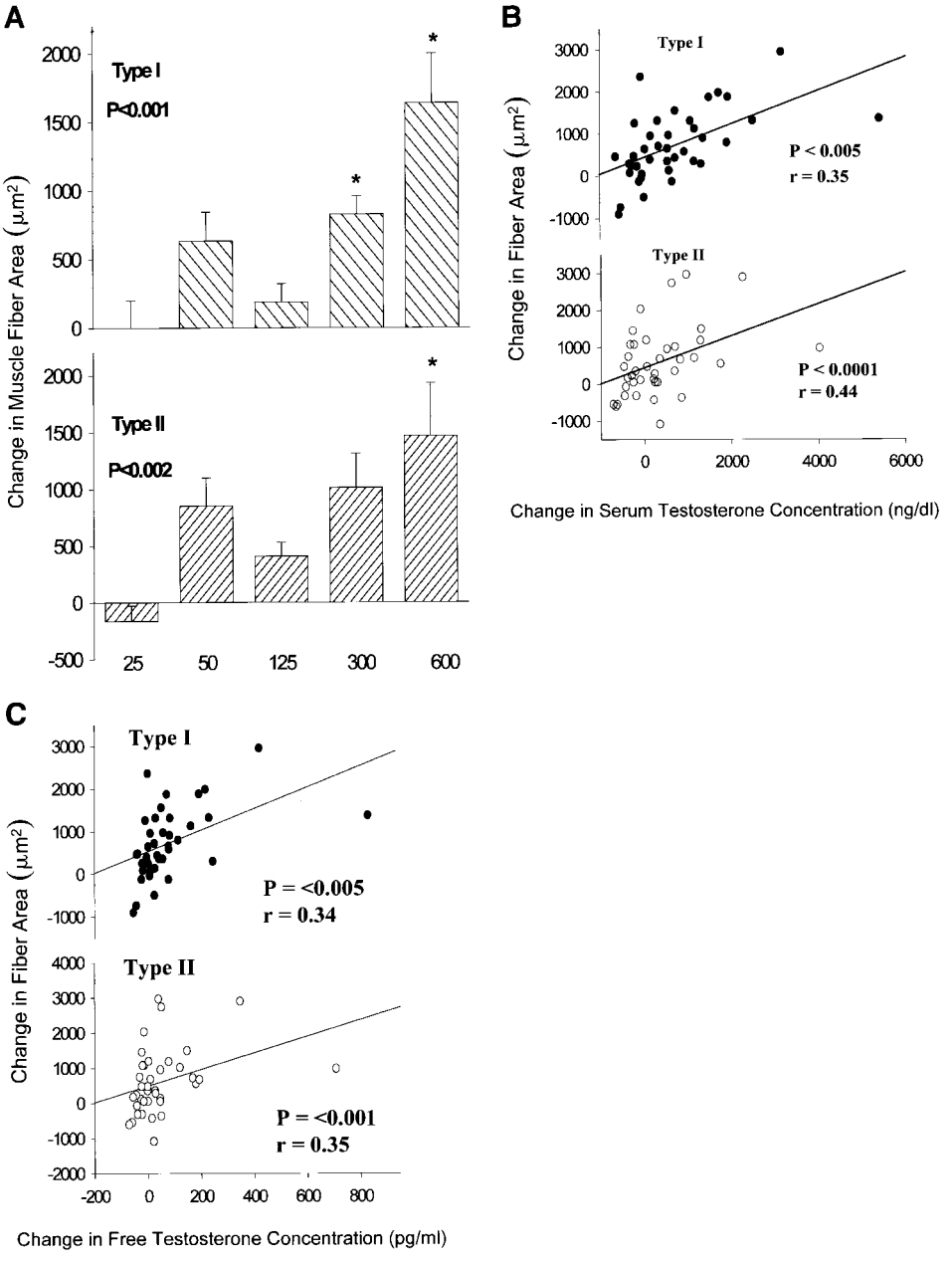

Fig. 1. *A*: change in cross-sectional areas of type I and type II skeletal muscle fibers before and after 20 wk of treatment with gonadotropin-releasing hormone (GnRH) agonist and graded doses of testosterone. Data are means ± SE. *Significantly different from 0. *B*: correlation between change in serum total testosterone concentrations during treatment and change in cross-sectional area of type I (*top*) and type II (*bottom*) muscle fibers. *C*: correlation between change in serum free testosterone concentrations during treatment and change in cross-sectional area of type I (*top*) and type II (*bottom*) muscle fibers.

experienced a significant increase in cross-sectional area of type I fibers (Figs. 1*A* and 2). Similarly, men receiving the highest dose of testosterone enanthate demonstrated a significant increase in the cross-sectional area of type II fibers. Figure 2 illustrates the change in muscle fiber cross-sectional area in a representative subject, who received the 600-mg testosterone dose.

The changes in cross-sectional areas of both type I and type II muscle fibers were significantly correlated with total [$r = 0.35$ ($P < 0.005$) for type I fibers; $r = 0.44$ for type II fibers ($P < 0.0001$)] and free testosterone [$r = 0.34$ ($P < 0.005$) for type I muscle fibers; $r = 0.35$ ($P < 0.001$) for type II muscle fibers] concentrations during treatment (Table 4; Fig. 1, *B* and *C*). The percent increases in mean cross-sectional areas of type I fibers were numerically greater than those in type II fibers in men receiving the 300-mg dose (%increase: 33 ± 6 vs. 24 ± 11 in type I and type II fiber cross-sectional area, respectively, $P = 0.190$) and the 600-mg dose (%increase: 51 ± 14 vs. 39 ± 13 in type I and type II fibers, respectively, $P = 0.384$); however, the differences in the increase in mean fiber cross-sectional areas did not achieve statistical significance.

Muscle fiber composition. The relative proportions of type I and type II muscle fibers at baseline were not significantly different among the five treatment groups (Fig. 3). Administration of GnRH agonist plus testosterone enanthate did not significantly change the number of type I or type II fibers per unit area in the cross sections that were examined at any of the testosterone doses. Similarly, there was no significant change from

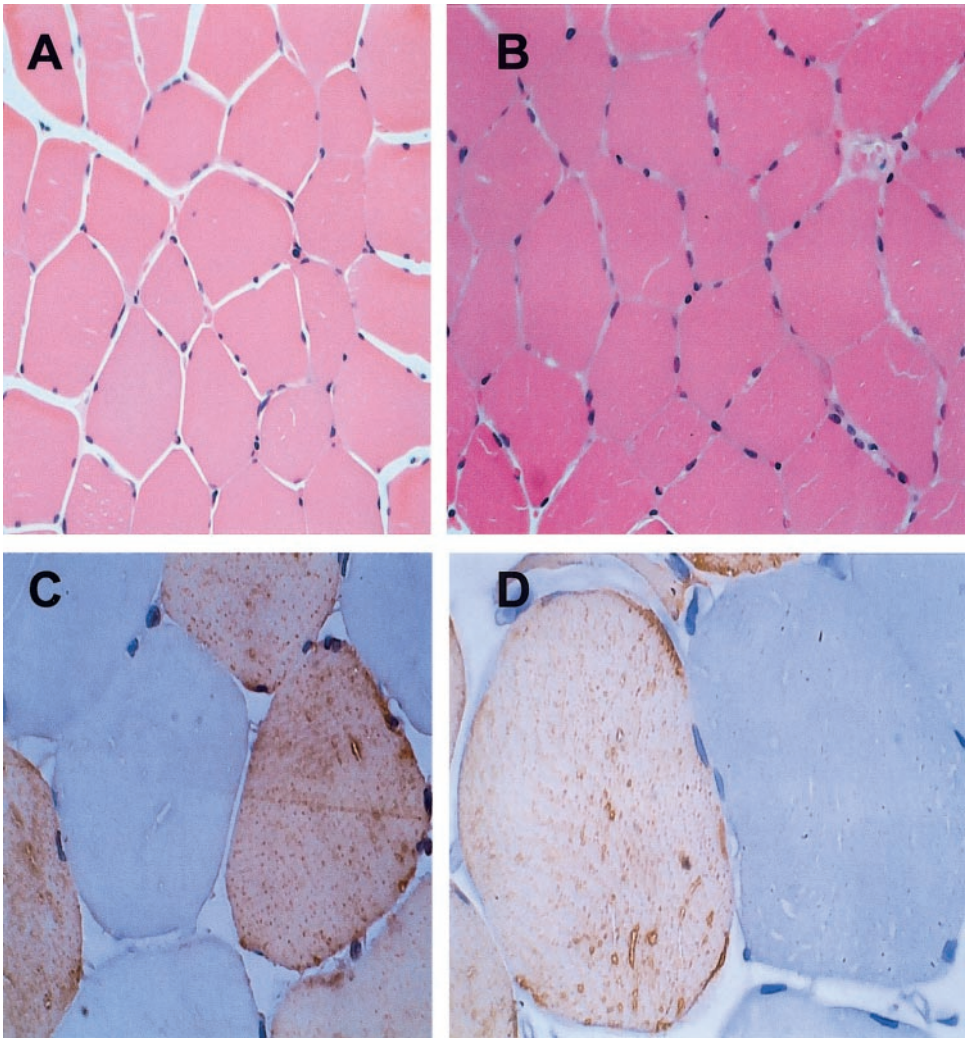

Fig. 2. Cross sections of muscle biopsies before and after 20 wk of treatment in one man treated with GnRH agonist and 600 mg of testosterone enanthate weekly. *A* and *C*: baseline sections; *B* and *D*: sections obtained after 20 wk of treatment. The magnification is 200-fold in *A* and *B* and 1,000-fold in *C* and *D*.

baseline in the relative proportion of type I and type II fibers in any treatment group (Fig. 3).

The concentration of mRNAs for MHC isotypes, I, IIa, IIb, and IIx did not significantly change relative to GAPDH mRNA or the amount of 28S RNA (Table 5; Fig. 4).

Myonuclear number. The average number of myonuclei per muscle fiber was not significantly different among the five treatment groups at baseline. Administration of testosterone enanthate was associated with a dose-dependent increase in myonuclear number. In men receiving the 300-mg weekly dose of testosterone enanthate, the mean ± SE myonuclear number increased from 3.2 ± 0.2 to 3.9 ± 0.2 per type I fiber (P = 0.001) and from 3.3 ± 0.2 to 3.7 ± 0.2 per type II fiber (P = 0.118); similarly, myonuclear number increased significantly from 3.4 ± 0.2 to 4.2 ± 0.2 per type I fiber (P = 0.028) and from 2.9 ± 0.3 to 4.2 ± 0.2 per type II

Table 4. *Correlations of changes in skeletal muscle fiber morphometric measures with changes in serum total and free testosterone concentrations*

Outcome Measure	Correlation with Change in Total Testosterone	Correlation with Change in Free Testosterone
Change in mean CSA type II fibers, μm^2	0.42 (<0.01)	0.35 (<0.05)
Change in mean CSA type I fibers, μm^2	0.57 (<0.001)	0.34 (<0.005)
Change in percent type II fibers	0.06 (NS)	0.006 (NS)
Change in percent type I fibers	0.07 (NS)	0.02 (NS)
Change in myonuclear number, type II fibers	0.42 (<0.01)	0.40 (< 0.01)
Change in myonuclear number, type I fibers	0.17 (NS)	0.13 (NS)

CSA, cross-sectional area. Change in serum total and free testosterone concentrations was calculated as the difference between *week 16* and baseline values. Change in other morphometric measures represents the difference between *week 20* and baseline values. Changes in muscle morphometric outcome measures were correlated with changes in total and free testosterone concentrations. The numbers in parentheses indicate P values.

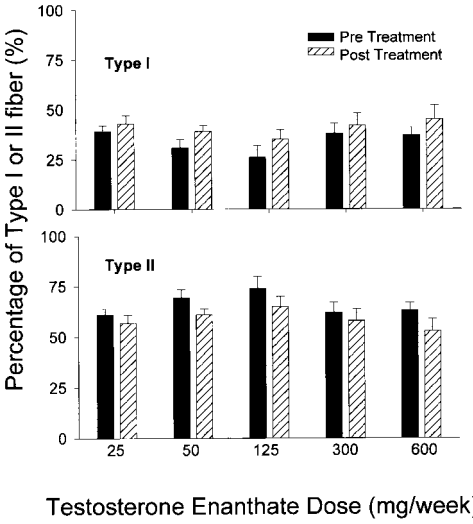

Fig. 3. Effects of treatment with GnRH agonist and graded doses of testosterone on relative proportion of type I and type II skeletal muscle fibers in healthy young men. Mean ± SE percent type I and II skeletal muscle fibers in biopsies obtained at baseline before initiation of treatment (pretreatment, filled bars) and after 20 wk of treatment (posttreatment, hatched bars) are shown. The percentage of type I muscle fibers is shown in *top*, and percentage of type II fibers is shown in *bottom*. None of the posttreatment values was significantly different from baseline values.

fiber ($P = 0.017$) in men receiving the 600-mg dose (Fig. 5, *A* and *B*). The muscle fiber cross-sectional area was significantly correlated with the myonuclear number (Fig. 5*B*). The number of myonuclei per unit fiber area did not significantly change in any of the treatment groups.

Blood capillary density. The capillary density, defined as the number of blood capillaries per unit muscle area, was not significantly different among the five treatment groups at baseline (Table 6). The change in capillary density after testosterone treatment was not significantly correlated with testosterone dose or concentration and did not significantly differ among the five groups. The capillary-to-muscle fiber ratio also did not differ among the five groups.

DISCUSSION

In healthy young men in whom testicular testosterone production had been suppressed by a GnRH agonist administration of graded doses of testosterone was associated with dose-dependent changes in circulating concentrations of total and free testosterone (11). We have previously reported, using this Leydig cell clamp model, that testosterone dose-dependently increases fat-free mass and muscle size (11). Data presented in this report demonstrate that testosterone-induced gains in muscle size were associated with a significant increase in muscle fiber cross-sectional area. The cross-sectional areas of both type I and type II fibers increased in proportion to testosterone concentrations. The relative proportion of type I and type II fibers did not change significantly. We therefore conclude that testosterone increases skeletal muscle size primarily by inducing muscle fiber hypertrophy.

This study constitutes the first demonstration that androgens induce skeletal muscle fiber hypertrophy in healthy young men. These data generated from skeletal muscle of healthy young men are consistent with data from rat studies that reported an increase in fiber diameter in levator ani muscle of androgen-treated rats (22, 44, 45).

The adaptive response of the skeletal muscle varies with the type of stimulus, the fiber composition of the muscle being studied, and the time point at which the biopsies are taken. For instance, muscle stretch in wing muscles of birds results in an early increase in muscle fiber numbers (2), but the hypertropic and hyperplastic responses of the skeletal muscle have different time courses. In this study, muscle biopsies were obtained only at one time point after testosterone treatment. Also, different muscle groups may respond differently to anabolic stimuli, depending in part on the fiber composition. We studied only one muscle

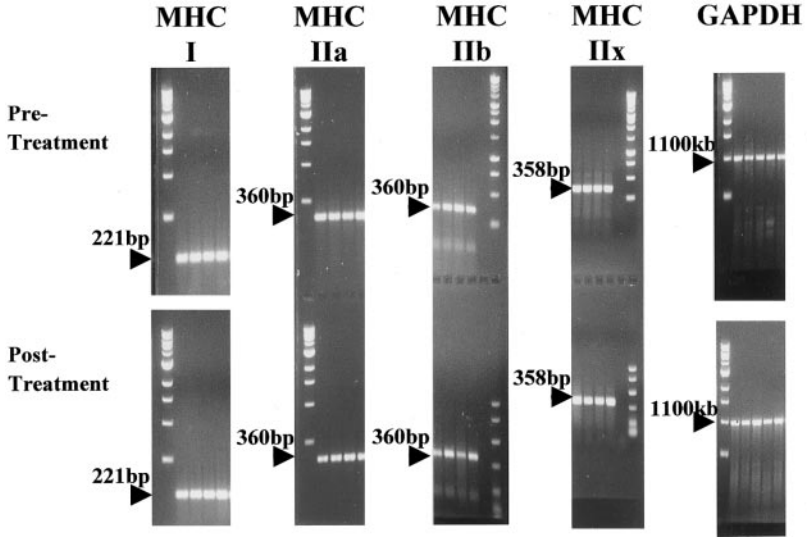

Fig. 4. Representative electrophoretic gels demonstrating the DNA bands resulting from RT-PCR of mRNAs of myosin heavy chain (MHC) isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Top* gels represent baseline samples from 4 randomly selected men from different treatment groups. *Bottom* gels represent posttreatment samples from the same subjects.

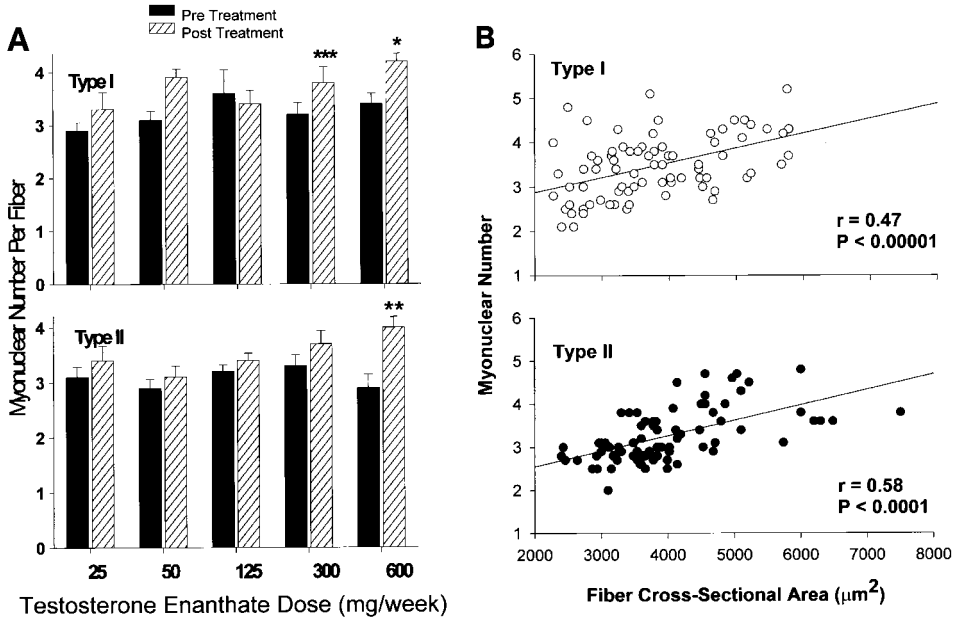

Fig. 5. *A*: effects of treatment with GnRH agonist and graded doses of testosterone on skeletal muscle fiber myonuclear number in healthy young men. Mean ± SE numbers of myonuclei in muscle fibers in baseline biopsies are shown in filled bars, those in biopsies obtained after 20 wk of treatment in hatched bars. *Top*: number of myonuclei in type I muscle fibers; *bottom*: type II muscle fibers. $*P = 0.028$ vs. baseline; $**P = 0.017$; $***P < 0.001$. *B*: correlation between the change in myonuclear number and the change in cross-sectional areas of type I (*top*) and type II (*bottom*) muscle fibers.

group; we do not know whether these findings can be generalized to other muscle groups with different fiber composition.

As discussed by Allen and Edgerton (1), muscle hypertrophy involves the addition of newly formed myonuclei via the fusion of myogenic cells to the adult myofibers. There is substantial evidence supporting a role for the modulation of myonuclear number during muscle remodeling in response to injury or disease (1, 25). In our study, the myonuclear number increased in direct relation to the increase in muscle fiber diameter. Therefore, it is possible that muscle fiber hypertrophy and increase in myonuclear number were preceded by testosterone-induced increase in satellite cell number and their fusion with muscle fibers. The hypertrophy of levator ani muscle in the female rat induced by exogenous testosterone administration is associated with satellite cell proliferation (32). Muscle remodeling and repair after injury often involve satellite cell replication and recruitment of new stem cells into the myogenic cell lineage (38). The mechanisms by which testosterone might increase satellite cell number are not known. An increase in satellite cell number could occur by an increase in satellite cell replication, inhibition of

Table 5. mRNA*concentrations of MHC isotypes I, IIa, IIb, and IIx before and after treatment with graded doses of testosterone in healthy young men*

	Dose, mg				
	25	50	125	300	600
			MHC type I		
Baseline	1.9 ± 0.3	2.2 ± 0.2	2.2 ± 0.4	1.7 ± 0.3	1.5 ± 0.1
Week 20	2.2 ± 0.2	2.0 ± 0.2	2.2 ± 0.1	2.0 ± 0.1	2.0 ± 0.2
n	4	3	4	4	4
			MHC type IIa		
Baseline	1.9 ± 0.2	2.3 ± 0.3	2.3 ± 0.3	1.7 ± 0.2	1.5 ± 0.1
Week 20	2.2 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.1 ± 0.2
n	4	3	4	4	4
			MHC type IIb		
Baseline	1.2 ± 0.1	1.3 ± 0.3	1.3 ± 0.1	1.3 ± 0.3	1.0 ± 0.1
Week 20	1.2 ± 0.4	2.0 ± 0.3	1.8 ± 0.2	1.6 ± 0.3	1.2 ± 0.3
n	4	3	4	4	4
			MHC type IIx		
Baseline	2.5 ± 0.3	2.7 ± 0.3	2.9 ± 0.4	2.1 ± 0.3	1.8 ± 0.1
Week 20	2.7 ± 0.2	2.4 ± 0.2	2.8 ± 0.1	2.4 ± 0.2	2.5 ± 0.2
n	4	3	4	4	4

Data are means ± SE. Relative concentrations of myosin heavy chain (MHC) isoform mRNAs, measured by RT-PCR, are expressed as ratios of the optical densities of MHC isoform mRNA and glyceraldehyde-3-phosphate dehydrogenase mRNA. Changes from baseline scores were not significantly different for any of the outcome measures listed above.

Table 6. *Absolute and relative blood capillary density bevore and after treatment with GnRH agonist and graded doses of testosterone*

	Dose, mg				
	25	50	125	300	600
	Capillary density per unit area (overall ANOVA, P = 0.49)				
Baseline	17 ± 2	21 ± 2	22 ± 2	18 ± 1	18 ± 1
Week 20	19 ± 2	22 ± 2	20 ± 1	21 ± 2	22 ± 1
	Capillary density per muscle fiber (capillary-to-fiber number ratio, P = 0.08)				
Baseline	1.0 ± 0.2	1.1 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.2
Week 20	1.1 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.8 ± 0.2	2.1 ± 0.2

Data are means ± SE. Unit area = 600 μm^2. GnRH, gonadotropin-releasing hormone. None of the *week 20* values was significantly different from the corresponding baseline values for any of the outcome measures listed above.

satellite cell apoptosis, and/or increased differentiation of stem cells into the myogenic lineage. We do not know which of these processes is the site of regulation by testosterone. The hypothesis that testosterone promotes muscle fiber hypertrophy by increasing the number of satellite cells should be further tested. Because of the constraints inherent in obtaining multiple biopsy specimens in humans, the effects of testosterone on satellite cell replication and stem cell recruitment would be more conveniently studied in an animal model.

Although we did not find a significant change in muscle fiber number per unit muscle area in the cross sections that were examined at any dose of testosterone, we recognize that the sampling was limited to a small region, and small but significant changes in muscle fiber number in other regions night have been missed. Additionally, the muscle fibers in the human vastus lateralis muscle do not run the entire length of the muscle; therefore, it is not possible to accurately measure the absolute number of muscle fibers in this muscle. The number of muscle cells that stained for proliferating cell nuclear antigen was too small to permit accurate measurement of change (data not shown).

Testosterone administration did not significantly affect the relative proportion of type I and type II fibers. Other anabolic hormones and interventions have been reported to affect fiber type transition (reviewed in Ref. 36). In the rat, hypothyroidism causes a shift of fast-twitch to slow fibers, whereas hyperthyroidism causes a shift in the opposite direction (29, 33). Analogous effects of thyroid hormone have been demonstrated in human muscles (13, 37). The administration of growth hormone in the rat increases muscle mass and also the relative proportion of type I fibers (5, 6). Testosterone treatment of female rats is associated with a decrease in the percentage of type I fibers in the gastrocnemius, extensor digitorum longus, and soleus muscles (22). High-intensity resistance training in previously untrained men induced an MHC IIb-to-MHC IIa transition (36). Other studies have reported that resistance exercise increases the ratio of type II to type I fiber area in biceps brachii, indicating preferential hypertrophy of type II fibers (31). We did not observe a change in the relative proportion of type I and type II fibers. The mRNA concentrations of type I, IIa, IIB, and IIx MHC isotypes did not change after treatment in any group. Because the number of men in whom tissue was available for these analyses was limited, it is possible that the sample did not have sufficient power to detect small differences in the relative proportions of MHC isotypes.

There are some similarities and many notable differences in the skeletal muscle response to testosterone and resistance exercise training. Both testosterone and resistance exercise training increase the cross-sectional areas of both type I and II fibers (31). Resistance exercise in cats leads to the appearance of split fibers (20), which we did not see in longitudinal sections of the muscle biopsies even at the highest dose of testosterone. It is possible that the fiber splitting previously reported in cats with resistance exercise (20) was the result of mechanical loading of the muscle with relatively heavy loads, a stimulus for muscle fiber change that is not replicated by testosterone administration. Resistance exercise increases the ratio of type II to type I fiber area (31), but testosterone does not affect fiber type transition and induces the hypertrophy of both types I and II fibers. These data suggest that, although testosterone and resistance exercise training both increase muscle size, they likely do so by different mechanisms. We recognize that the effects of resistance exercise training vary with the intensity, mode, and frequency of exercise stimulus; therefore, the comments above may apply only to the specific regimens of resistance exercise that were studied.

In summary, our data demonstrate that the increase in skeletal muscle mass during short-term testosterone administration is associated with dose-dependent increases in muscle fiber cross-sectional area and myonuclear number. The hypothesis that testosterone increases the number of satellite cells, whose fusion with the muscle fibers leads to muscle fiber hypertrophy and increase in myonuclear number, should now be studied.

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