Angiotensin-converting enzyme inhibition attenuates myonuclear addition in overloaded slow-twitch skeletal muscle

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Westerkamp, Christopher M., and Scott E. Gordon. Angiotensin-converting enzyme inhibition attenuates myonuclear addition in overloaded slow-twitch skeletal muscle. Am J Physiol Regul Integr Comp Physiol 289: R1223–R1231, 2005. First published June 16, 2005; doi:10.1152/ajpregu.00730.2004.—Because optimal overload-induced skeletal muscle hypertrophy requires ANG II, we aimed to determine the effects of blocking ANG II production [via angiotensin-converting enzyme (ACE) inhibition] on potential mediators of hypertrophy in overloaded skeletal muscle, namely, myonuclear addition and fibroblast content. In a 2 × 2 design, adult (200–225 g) female Sprague-Dawley rats were placed into one of four groups (n = 8/group): 7-day skeletal muscle overload, sham operation, 7-day skeletal muscle overload with ACE inhibition, or sham operation with ACE inhibition. Functional overloads of the plantaris and soleus muscles were produced via bilateral surgical ablation of the synergistic gastrocnemius muscle, and ACE inhibition was accomplished by the addition of the ACE inhibitor enalapril maleate to the animals’ daily drinking water (0.3 mg/ml). Myonuclear addition and extrasarcolemmal nuclear proliferation, as measured by in vivo 5-bromo-2′-deoxyuridine labeling, were significantly (P < 0.05) increased by overload in both the slow-twitch soleus and fast-twitch plantaris muscles. Furthermore, ACE inhibition attenuated these overload-induced increases in the soleus muscle but not in the plantaris muscle. However, the effect of ACE inhibition on soleus extrasarcolemmal nuclei was not likely due to differences in fibroblast content because overload elicited significant increases in vimentin-positive areas in soleus and plantaris muscles, and these areas were unaffected by ACE inhibition in either muscle. There was no effect of ACE inhibition on any measure in sham-operated muscles. Collectively, these data indicate that ANG II may mediate the satellite cell response to overload in slow-twitch soleus but not in fast-twitch plantaris muscles and that this effect may occur independently of changes in fibroblast content.

SKELETAL MUSCLE MASS INCREASES from loading in humans (26) and animal models (19, 49). Satellite cells, which are myoblasts lying between the basalm lamina and sarcolemma of mature myofibers, are vital to skeletal muscle hypertrophy. In response to skeletal muscle loading, myonuclear addition occurs from satellite cells activating, proliferating, differentiating, and fusing with mature myofibers to maintain a fairly constant myonuclear domain as protein accretion progresses (1, 23). The importance of this phenomenon is demonstrated by the fact that blocking satellite cell proliferation via irradiation can completely prevent overload-induced skeletal muscle hypertrophy (44). Hormonal input and the increased mechanical loading itself both appear to be essential for optimal satellite cell activation and overload-induced skeletal muscle hypertrophy (3, 11). Presently, at least 18 hormones and growth factors are known to be involved in satellite cell activity (23).

Although it has not previously been examined with respect to satellite cell activity or myonuclear addition, the hormone ANG II is necessary for optimal overload-induced skeletal muscle hypertrophy (19). Inhibiting angiotensin-converting enzyme (ACE), the enzyme that converts ANG I to ANG II, has been shown to attenuate slow-twitch soleus and fast-twitch plantaris muscle hypertrophy by 96 and 57%, respectively, over 28 days of overload in rats (19). Furthermore, perfusing ANG II directly onto overloaded soleus muscles in a separate set of animals receiving ACE inhibitors rescued back most of this lost hypertrophic response (19). Also, the ACE deletion allele genotype (conferring greater ACE activity) is associated with a greater hypertrophic response to functional overload in human skeletal muscle (17). These findings suggest the importance of the renin-angiotensin system in skeletal muscle hypertrophy. ANG II and overload appear to act synergistically to induce skeletal muscle hypertrophy because neither ACE inhibition nor ANG II administration affects nonoverloaded muscle mass (19). Although the mechanism of ANG II action in overloaded, hypertrophying skeletal muscle is unclear, we postulated that this mechanism may at least partly involve satellite cell proliferation because of the mitogenic effect of ANG II on smooth muscle cells. For instance, ANG II mediates smooth muscle cell proliferation after vascular injury (40). ANG II also stimulates smooth muscle cell proliferation to a greater extent in hypertensive compared with normotensive rats (38), indicating a synergism between mechanical loading and ANG II on smooth muscle cell proliferation similar to that seen in overload-induced skeletal muscle hypertrophy (19).

In addition to overload-induced skeletal muscle hypertrophy and smooth muscle cell proliferation, ANG II also mediates overload-induced cardiac hypertrophy (6). This is likely due to the synergistic effect of loading and ANG II directly on cardiac myocytes (45) along with the proliferative effect of ANG II on cardiac fibroblasts in the overloaded hypertrophying heart (35), which contribute to cardiac myocyte hypertrophy via paracrine growth factor secretion (21). Interestingly, skeletal muscle fibroblasts mediate skeletal myoblast proliferation and differentiation also via paracrine growth factor secretion (42). Moreover, mechanically loaded fibroblasts are known to modify the extracellular matrix (27), the composition of which is also an important mediator of skeletal myoblast proliferation and differentiation (25, 36). Evidence of fibroblast activation and
proliferation has been observed after 1 wk of chronic overload in rat skeletal muscle (22); however, it is unknown whether ANG II plays a role in this overload-induced skeletal muscle fibroblast activity. We postulated that ANG II-mediated hypertrophy of overloaded skeletal muscle acts in part via control of fibroblast content in a manner similar to overload-induced cardiac hypertrophy. Furthermore, due to the mitogenic effect of fibroblasts on skeletal muscle myoblasts, we also speculated that any differences in myonuclear addition due to ACE inhibition occur concomitant to potential differences in fibroblast content in overloaded skeletal muscle.

The primary and secondary purposes of this investigation were to examine the effects of blocking ANG II production (via ACE inhibition) on myonuclear addition and fibroblast content, respectively, in overloaded skeletal muscle. We hypothesized that ACE inhibition would attenuate overload-induced myonuclear addition and increases in fibroblast content during 1 wk of overload in rat slow-twitch soleus and fast-twitch plantaris muscles. We found that ACE inhibition attenuated overload-induced myonuclear addition in the slow-twitch soleus muscle but not in the fast-twitch plantaris muscle. However, contrary to our hypothesis, ACE inhibition did not attenuate overload-induced increases in fibroblast content in either muscle.

METHODS

Experimental design. The primary and secondary purposes of this investigation were to examine the effect of blocking ANG II production on myonuclear addition and fibroblast content, respectively, in overloaded skeletal muscle. Using a 2 × 2 design (skeletal muscle overload × ACE inhibition), we examined the soleus and plantaris muscles from all experimental animals for myonuclear addition and fibroblast content after 7 days of overload induced by gastrocnemius ablation. Soleus and plantaris muscle cross sections were immunohistochemically detected for dystrophin, the nucleotide analog 5-bromo-2′-deoxyuridine (BrdU; continuously infused via osmotic pump during the 7-day overload period) to detect myonuclear addition, and vimentin to detect fibroblast content. A 7-day overload period was chosen because nuclear proliferation is highest within the first week of skeletal muscle overload using the synergist ablation model (46).

Experimental animals. Female Sprague-Dawley rats weighing 200–225 g were weight matched and placed into one of four groups (n = 8/group): sham operated (S), sham operated with ACE inhibitor (SA), skeletal muscle overload (O), and skeletal muscle overload with ACE inhibitor (OA). All animals were housed at the animal care facility in the Brody School of Medicine at East Carolina University. Animals were housed individually, given standard rat chow and water ad libitum, and kept on a 12:12-h light-dark cycle. This study was approved by the East Carolina University Animal Care and Use Committee.

ACE inhibitor treatment. Rats in the SA and OA groups had the ACE inhibitor enalapril maleate (Sigma, St. Louis, MO) added fresh daily to their drinking water at a concentration of 0.3 mg/ml starting 2 days before skeletal muscle overload surgery and continuing throughout the full experimental treatment. Water intake was measured daily, and ACE inhibitor dosage was calculated to be 37 mg·kg body wt⁻¹·day⁻¹. Gordon et al. (19) previously showed that this dosage of ACE inhibitor (~35 mg·kg body wt⁻¹·day⁻¹) prevents 96% of soleus muscle and 57% of plantaris muscle hypertrophy induced by 28 days of overload after gastrocnemius muscle ablation in female Sprague-Dawley rats of the same age.

Skeletal muscle overload surgery. Rats in the skeletal muscle overload groups underwent bilateral ablation of gastrocnemius muscles according to procedures previously described (19), which consisted of the removal of both heads of the gastrocnemius muscles to simultaneously overload the soleus and plantaris muscles. This is an ideal model with which to compare fiber-type-specific responses because the rat soleus and plantaris muscles are 89% slow twitch by mass and 93% fast twitch by mass, respectively (5). Briefly, all animals were first anesthetized with an intraperitoneal injection of a cocktail consisting of ketamine and xylazine (80 and 9 mg/kg body wt, respectively), and the hindlimbs and a section of the back were shaved of hair and scrubbed with iodine swabs. Then, under aseptic conditions, an incision was made through the skin and biceps femoris muscle along the posterior longitudinal line of both hindlimbs. For the animals in the O and OA groups, the lateral and medial heads of both gastrocnemius muscles were separated from the soleus and plantaris muscles at the Achilles tendon. Both gastrocnemius heads were severed at the Achilles tendon, and the distal two-thirds of the muscle was removed without avoiding damage to underlying tissue. The animals in the S and SA groups had similar surgery, without cutting of the Achilles tendon or excision of the gastrocnemius muscle. Incisions were then closed with wound clips and checked daily for signs of infection. During recovery from surgery, all animals received a one-time subcutaneous injection of the analgesic buprenorphine (0.03 mg/kg body wt). Animals were ambulatory within 3–5 h after surgery and remained ambulatory throughout the 7-day experimental period. No postoperative complications or infections were noted from surgical treatment.

Osmotic pump implant and in vivo nuclear labeling. During the skeletal muscle overload surgery, each rat also had a miniosmotic pump (Alzet model 2ML1, Alza, Cupertino, CA) inserted subcutaneously on the back that contained 25 mg/ml BrdU (Sigma) in sterile saline. BrdU is a thymidine analog incorporated into proliferating nuclei during the S phase of the cell cycle. The Alzet model 2ML1 delivers a 10 µl/h flow rate for a 7-day period, thus delivering ~25–30 mg of BrdU·kg body wt⁻¹·day⁻¹ in this investigation. With a similar continuous dose of BrdU, a 7-day period has also been used to demonstrate myonuclear addition in the skeletal muscle of endurance-trained rats (48).

Tissue harvesting. Seven days after the initial surgery, animals were weighed and anesthetized with an intraperitoneal injection of ketamine-xylazine (80 and 9 mg/kg body wt, respectively) and euthanized by decapitation. Trunk blood was collected for later measurement of serum ACE activity. The soleus and plantaris muscles from both hindlimbs were quickly excised and trimmed of excess fat and connective tissue. Muscles were weighed and then snap frozen in isopentane chilled in liquid nitrogen for ~1–2 min. These samples were stored at ~80°C until further analysis. To normalize muscle wet weight to tibia length and account for differences in skeletal growth of the animals, the right tibias of all animals were removed and the lengths were measured with a caliper.

The blood samples (~3 ml) were allowed to clot on ice and centrifuged at 1,500 g for 20 min at 4°C, after which the serum was separated, frozen in liquid nitrogen, and stored at ~80°C. Serum samples were later measured for serum ACE activity (Sigma diagnostics kit 305-UV). As expected, animals in the SA and OA groups had significantly lower serum ACE activity (26.52 ± 8.24 and 25.40 ± 6.58 U/l, respectively) vs. animals in the S and O groups (87.94 ± 17.14 and 103.29 ± 27.64 U/l, respectively). These results are similar to those previously observed by Gordon et al. (19).

Muscle protein determination. A section from the midbellies of the right soleus and plantaris muscles from each animal was obtained on day 1 of this investigation. Tissue sections were weighed and homogenized as a 3.5% (wt/vol) solution in a buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 100 mM β-glycerophosphate, 15 mM Na2HPO4·10H2O, 25 mM NaF, 5 mM Na3VO4, 50 µg/ml leupeptin, 50 µg/ml pepstatin, and 33 µg/ml aprotonin. Homogenizations were completed on ice in a ground glass homogenizer using a variable speed motor. A modified Lowry procedure (DC Lowry protein assay; Bio-Rad, Hercules, CA) was used to assess
Anti-dystrophin and anti-vimentin immunohistochemical stain. An anti-dystrophin stain was used to visualize the sarcolemma of mature myocytes in muscle cross sections, which allowed for the delineation of nuclei on either side of the muscle fiber membrane as well as measurement of fiber area. In separate cross sections on separate slides, an anti-vimentin stain was used to visualize skeletal muscle fibroblasts. Vimentin is a structural protein that is considered to be a marker for fibroblasts (18) and has been previously used to assess increases in fibroblast content in skeletal muscle cross sections after laceration (12) or chronic low-frequency electrical stimulation (41).

Commercially available monoclonal mouse antibodies for dystrophin (NCL-DYS-2; Novacastra Laboratories, via Vector Laboratories, Burlingame, CA) or vimentin (clone LN-6, catalog no. V2258; Sigma) were used along with an anti-mouse secondary antibody and amplification system (Vectastain Elite ABC kit, Vector Laboratories) according to the modified methods of Dupont-Versteegden et al. (14). We obtained 10-μm serial cross sections of the midbellies of the left soleus and plantaris muscles from each animal using a cryostat microtome at −20°C. These were subsequently placed on uncoated microscope slides for anti-dystrophin staining and poly-L-lysine-coated slides for anti-vimentin staining. All immunohistochemical steps were performed at room temperature unless otherwise noted. Sections were rehydrated in PBS for 10 min, blocked for 60 min with 10% normal horse serum in PBS, and then rinsed twice for 2 min in PBS. Next, the primary antibodies were applied at a 1/4 dilution (anti-dystrophin) for 30 min or 1/400 dilution (anti-vimentin) for 60 min in PBS with 1% normal horse serum and then washed twice for 2 min each in PBS. The sections were then incubated for 10 min in a biotinylated anti-mouse secondary antibody at 1/200 dilution (dystrophin sections) or in a rat adsorbed anti-mouse biotinylated secondary antibody at 1/100 dilution (vimentin sections) and again washed twice for 2 min in PBS. Next, the sections were incubated for 5 min with an avidin-biotinylated enzyme complex reagent solution and subsequently washed twice for 5 min in PBS. A commercially available coloring solution containing 0.02% H2O2 and 0.04% 3,3′-diaminobenzidine (diaminobenzidine substrate kit, Vector Laboratories) was then applied to the sections for ~5 min. Sections were then washed for 5 min in distilled H2O, placed in 100% methanol for 5 min, and allowed to air dry for 10 min. Sections stained for vimentin were then mounted with Permount and coverslips, and sections stained for dystrophin were then counterstained for BrdU. For validation of the anti-vimentin staining specificity, a nonspecific mouse IgG (Vector Laboratories) was used on several separate muscle sections in place of, but at the same concentration as, the anti-vimentin primary antibody.

Anti-BrdU immunohistochemical stain. For sections in which the sarcolemma was identified by the anti-dystrophin stain, an anti-BrdU counterstain was employed to identify proliferating nuclei that had incorporated into the myofiber. Slides were rehydrated for 3 min in PBS and then incubated in 2 N HCl for 60 min at 37°C in a humid atmosphere and then neutralized with a 1× Tris-borate-EDTA buffer (pH 8.5) for 10 min at room temperature, changing the solution twice. Muscle sections were then permeabilized by incubation in PBS containing 0.02% Nonidet P-40 substitute (Sigma) for 10 min, changing the solution three times. Sections were then stained for BrdU by slightly modifying the procedures of a commercially available kit (BrdU labeling and detection kit II; Roche Diagnostics, Indianapolis, IN) and those of Dupont-Versteegden et al. (14). Sections were incubated with an anti-BrdU mouse monoclonal antibody in PBS for 30 min at 37°C in a humid atmosphere, after which they were washed three times in PBS containing 0.1% Nonidet P-40 substitute at room temperature. Next, the sections were covered with an anti-mouse alkaline phosphate-linked secondary antibody in PBS, incubated for 30 min at 37°C in a humid atmosphere, and then washed three times in PBS containing 0.1% Nonidet P-40 substitute at room temperature. Sections were then covered with a color substrate solution (nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate in 70% dimethylformamide) for 15 min and washed once with PBS. Sections were allowed to air dry and subsequently mounted with Permount and coverslips.

Image analysis. Before section analysis, the investigator performing the analysis was blinded as to the identity of the slides being analyzed. Muscle sections were viewed with a light microscope (Nikon Eclipse E400) mounted with a digital camera (Nikon Coolpix 990) interfaced with a personal computer with Sigma Scan Pro 5.0 software (SPSS, Chicago, IL). Images were taken at ×400 of four representative fields within each BrdU-dystrophin cross section of both muscles (soleus and plantaris) for each rat. From the four fields, a cumulative average total of 102.2 ± 3.0 (mean ± SE; range 70–137) muscle fibers per muscle section were measured for fiber area. Furthermore, the numbers of BrdU-positive subsarcolemmal and extrasarcolemmal nuclei (SS and ES, respectively) in these fields were counted along with the number of visible whole muscle fibers (WMFs) and muscle fibers partially in the field of view or partial muscle fibers (PMFs). These measures were used to calculate the total number of BrdU-positive SS and ES nuclei. A representative image demonstrating BrdU-positive SS and ES nuclei can be seen in Fig. 1. With this method, BrdU-positive SS nuclei are considered to measure satellite cells that had proliferated, differentiated, and fused with mature myofibers (14). The number of SS or ES nuclei in the fields was normalized to the total number of fibers in the field, where the total number of fibers in the field was considered to be WMF + PMF/2. The muscle fibers not completely in the field of view (PMFs; located on the field borders) were included in this measure because ES nuclei are not necessarily associated with one particular fiber. The assumption was made that PMFs averaged out to be 50% visible within the set of fields for any given muscle section. To validate this assumption, we were able to count SS nuclei located in either WMFs or the entire field (i.e., WMFs and PMFs) and normalize these numbers to WMF number or (WFM + PMF)/2, respectively. The two results over all 64 muscles were highly correlated (r = 0.97; P = 0.002), confirming the validity of this assumption. Thus all of the fibers in each image were counted when assessing SS and ES nuclear counts, whereas only completely visible WMFs were able to be used

Fig. 1. Representative photomicrograph of 1-wk overloaded soleus muscle cross section immunohistochemically stained for 5-bromo-2′-deoxyuridine (BrdU; blue) and dystrophin (brown). Filled arrows indicate BrdU-positive subsarcolemmal nuclei, and open arrows indicate BrdU-positive extrasarcolemmal nuclei.
when assessing fiber cross-sectional area. Images of four representative fields were taken of each vimentin-stained section at ×400, converted to a monochrome image, and corrected for background intensity using a clearfield image. An intensity threshold was then individually set for each image that encompassed all areas detected by the anti-vimentin stain outside of muscle fibers. This vimentin-positive area was calculated automatically by the imaging software. Vimentin-positive area was divided by (WMF + PMF/2) in the same manner as ES nuclei. Normalizing vimentin-positive area to muscle fiber number was necessary to eliminate any “dilution effect” due to differences in muscle fiber size between conditions.

Statistical analyses. Because this experiment was designed to determine the effect of overload and ACE inhibition on individuals and not to directly compare the responses between the muscles, separate two-way ANOVAs were performed on soleus and plantaris muscle results. A Fisher’s least significant difference test was used for any post hoc analyses (Statview; SAS Institute, Cary, NC). For analysis of body weight, a repeated-measures ANOVA was used. Statistical significance was set at \( P \leq 0.05 \).

RESULTS

Body weights. Animals were weight matched into four groups at the beginning of ACE inhibitor administration (2 days before overload or sham surgery; Table 1). At the time of surgery, there were no significant differences in body weight between any of the groups (data not shown). However, 7 days after surgery (at the time of death), the O and OA groups were slightly but significantly lower in body weight compared with the S group (4.3 and 4.9\%, respectively; see Table 1). This slight effect on body weight within the first week after overload surgery is similar to previous reports (19, 20).

Muscle wet weight, protein content, and fiber cross-sectional area. Soleus and plantaris muscle wet weights and protein contents were normalized to tibia length for all groups to account for the effects of skeletal and overall body growth during the experiment (see Table 1). Although overload increased soleus and plantaris muscle wet weights, ACE inhibition significantly attenuated this increase in wet weight by 29 and 39\% in overloaded soleus and plantaris muscles, respectively. In the plantaris muscle, overload elicited significant increases in muscle total protein content and fiber cross-sectional area as well, and these were also significantly attenuated by ACE inhibition (by 30 and 59\%, respectively). These results confirm previous findings of attenuated overload-induced plantaris muscle hypertrophy with ACE inhibition (19).

In contrast to the plantaris muscle, the soleus muscle did not demonstrate significant increases in muscle protein content or fiber cross-sectional area in response to overload. This is not necessarily surprising, as Ianuzzo and Chen (30) demonstrated that the increase in muscle wet weight can be much greater than that of protein content in the first several days after overload surgery. However, it has previously been shown that 28 days of overload greatly increases soleus muscle protein content in this model and that ACE inhibition attenuates this increase by 96\% (19). Although ACE inhibition significantly attenuated overload-induced soleus muscle wet weight hypertrophy in the present investigation, the 7-day overloading period was apparently too brief to observe overload-induced increases in soleus protein content or fiber cross-sectional area. However, it can take several weeks for changes in protein content to reflect changes in wet weight in the ablation model (31). Moreover, it has previously been shown that the attenuation of soleus muscle protein content hypertrophy is closely reflective of the attenuation of soleus muscle wet weight hypertrophy after 28 days of ACE inhibition and overload (19). Thus differences in soleus protein content with overload and ACE inhibition do appear to reflect changes in soleus wet weight over longer experimental treatment durations. There was no effect of ACE inhibition in sham-operated soleus or plantaris muscles in terms of muscle wet weight, protein content, or fiber cross-sectional area.

SS and ES nuclei. Within both the soleus and plantaris muscles, overload significantly increased BrdU-positive SS and ES nuclei vs. sham-operated muscles (see Figs. 2 and 3). Furthermore, ACE inhibition attenuated these increases in the soleus but not in the plantaris muscle. Albeit these were greatly reduced compared with those shown in the O group, significantly more BrdU-positive SS and ES nuclei were still retained in the OA soleus muscles compared with the S soleus muscles. In the sham-operated soleus and plantaris muscles, there was no effect of ACE inhibition on BrdU-positive SS or ES nuclei. Smith et al. (48) found no difference between SS myonuclei that were BrdU-positive and SS myonuclei that were MyoD positive, indicating that proliferating nuclei found within skeletal muscle fibers are most likely from satellite cell proliferation, differentiation, and incorporation. Thus BrdU-positive SS nuclei in this investigation were considered to be satellite cells.

Table 1. Body weights, tibia lengths, and indexes of muscle hypertrophy

<table>
<thead>
<tr>
<th></th>
<th>Sham Operated</th>
<th>Sham Operated With ACE-I</th>
<th>Overload</th>
<th>Overload With ACE-I</th>
</tr>
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<tbody>
<tr>
<td>Initial body weight, g</td>
<td>213.6 ± 3.6</td>
<td>212.9 ± 3.1</td>
<td>213.9 ± 3.2</td>
<td>213.4 ± 3.1</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>233.1 ± 4.4</td>
<td>229.3 ± 4.1</td>
<td>223.4 ± 4.4*</td>
<td>222.2 ± 3.5*</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>37.37 ± 0.19</td>
<td>37.50 ± 0.20</td>
<td>37.80 ± 0.30</td>
<td>37.65 ± 0.19</td>
</tr>
<tr>
<td>Soleus muscle</td>
<td></td>
<td></td>
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<tr>
<td>Wet weight, mg/mm</td>
<td>2.46 ± 0.11</td>
<td>2.55 ± 0.20</td>
<td>3.43 ± 0.28*</td>
<td>3.15 ± 0.21†</td>
</tr>
<tr>
<td>Total protein content, mg/mm</td>
<td>0.61 ± 0.04</td>
<td>0.56 ± 0.06</td>
<td>0.71 ± 0.02</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Fiber cross-sectional area, μm²</td>
<td>2,566.9 ± 128.7</td>
<td>2,619.0 ± 102.1</td>
<td>2,501.9 ± 207.9</td>
<td>2,613.8 ± 154.2</td>
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<tr>
<td>Plantaris muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wet weight, mg/mm</td>
<td>6.89 ± 0.47</td>
<td>7.04 ± 0.65</td>
<td>9.19 ± 0.73*</td>
<td>8.32 ± 0.61†</td>
</tr>
<tr>
<td>Total protein content, mg/mm</td>
<td>1.41 ± 0.07</td>
<td>1.41 ± 0.10</td>
<td>1.85 ± 0.23*</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>Fiber cross-sectional area, μm²</td>
<td>1,867.8 ± 133.9</td>
<td>1,687.1 ± 96.7</td>
<td>2,430.5 ± 121.9*</td>
<td>2,096.5 ± 118.4†</td>
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</tbody>
</table>

Values are means ± SE; \( n = 8 \) rats/group. Muscle wet weight and total protein content are normalized to tibia length. ACE-I, angiotensin-converting enzyme inhibition. Overload consisted of 7 days of soleus and plantaris muscle overload via gastrocnemius ablation. *\( P \leq 0.05 \) vs. sham-operated rat group; †\( P \leq 0.05 \) vs. overload rat group.
that had activated, proliferated, differentiated, and fused with mature myofibers.

**Skeletal muscle fibroblast content.** To examine whether ES nuclear differences reflected differences in fibroblast content, the vimentin-positive area was determined in muscle cross sections. There was a main effect of skeletal muscle overload on the vimentin-positive area (i.e., increased) in both the soleus and plantaris muscles (see Fig. 4), but ACE inhibition did not affect the vimentin-positive area in either overloaded or sham-operated soleus or plantaris muscles. In an effort to qualitatively assess whether some of the overload-induced increases in vimentin-positive area were potentially due to fibroblast proliferation, a double stain against both BrdU and vimentin was completed to observe BrdU-positive nuclei within vimentin-positive areas of some sham-operated and overloaded soleus and plantaris muscles (n = 3/group). It was observed that the number of BrdU-positive nuclei in vimentin-positive areas was consistently higher in overloaded vs. sham-operated conditions. A representative image demonstrating vimentin staining (Fig. 4, A and B) and vimentin and BrdU staining (Fig. 4D) in sham-operated and overloaded soleus muscle cross sections can be seen in Fig. 4.

**DISCUSSION**

Satellite cell activity is vital for overload-induced skeletal muscle hypertrophy because increases in skeletal muscle fiber area and mass generally require proportional increases in myonuclei (44). ANG II is known to mediate overload-induced skeletal muscle hypertrophy (19) and also interacts with overload to stimulate proliferation of other muscle cell types such as smooth muscle cells (38). Thus the primary purpose of this investigation was to examine the effect of blocking ANG II production (via ACE inhibition) on myonuclear addition in overloaded skeletal muscle. Furthermore, because skeletal muscle fibroblasts exert a paracrine influence on satellite cell proliferation and differentiation (42) and because ANG II may stimulate cardiac fibroblast proliferation in overloaded hearts (35), our secondary purpose was to determine the effect of...
blocking ANG II on fibroblast content in overloaded skeletal muscle. We found that ACE inhibition in rats significantly attenuated the increase in myonuclear addition and ES nuclear proliferation in overloaded slow-twitch soleus muscles but not in overloaded fast-twitch plantaris muscles. Surprisingly, the effect of ACE inhibition on ES nuclei was apparently not due to differences in fibroblast content because overload-induced increases in fibroblast content were not affected by ACE inhibition in either muscle. These data indicate that ANG II may mediate overload-induced slow-twitch muscle hypertrophy in part by mediating satellite cell activity and that this effect may be independent of changes in fibroblast content. However, ANG II may act through means other than satellite cell activity to mediate overload-induced fast-twitch muscle hypertrophy in this model.

As expected, myonuclear addition increased with overload in the soleus and plantaris muscles. This finding confirms similar reports by others using a compensatory model of skeletal muscle overload (49, 50), and it is known that numerous hormones are necessary for satellite cell activation, prolif-

![Fig. 4](image-url)
eration, and/or differentiation (23). Our results indicate that ANG II can also be included as a hormone that may mediate satellite cell activity, at least in overloaded slow-twitch muscle. However, it appears that the effects of ANG II are overload dependent because there was no effect of ACE inhibition on BrdU-positive nuclear number in sham-operated muscles. This effect is similar to the phenomenon whereby hindlimb irradiation blocks satellite cell activity in overloaded, but not in sham-operated, skeletal muscle (44). Our present data also fall in line with observations that ACE inhibition attenuates overload-induced skeletal muscle hypertrophy but does not cause atrophy in nonoverloaded muscles (Ref. 19 and present study). Similar to satellite cells, smooth muscle cells may also be more responsive to ANG II under conditions of mechanical loading because ANG II-stimulated smooth muscle proliferation is greater in hypertensive than in normotensive rats (38). Thus satellite cells in overloaded slow-twitch skeletal muscle may now be included with other muscle cell types such as smooth muscle cells (13) and cardiomyocytes (19) as being potential target cells for ANG II.

It is impossible to determine from our data exactly how ANG II mediates satellite cell activity because the use of BrdU and dystrophin staining limits the scope of this study to measuring nuclei that had activated, proliferated, differentiated, and fused to mature myofibers. The attenuated increase in SS BrdU-positive nuclei with ACE inhibition in overloaded soleus muscles may have resulted from an effect on any of the above factors. Apoptosis or necrosis of proliferating satellite cells before their fusion with myofibers also cannot be ruled out. Lastly, it has been suggested that there may be a population of satellite cells that activate, differentiate, and fuse to mature myofibers without proliferating during muscle regeneration (43). Such a population would not be assessed by BrdU incorporation methods. However, if this population exists, it likely contributes little to hypertrophy in overloaded skeletal muscle because blocking satellite cell proliferation completely prevents overload-induced muscle hypertrophy (44). Regardless of the mechanism(s) by which ACE inhibition affects satellite cell activity in overloaded slow-twitch muscle, the end result is an attenuation of myonuclear addition, a phenomenon essential for skeletal muscle hypertrophy (1).

It is unclear from our results whether ANG II acts directly on satellite cells to stimulate their proliferation, but this possibility is supported by the fact that ANG II stimulates early G1 phase cell cycle progression in immortalized skeletal myoblasts. In cultured C2C12 skeletal myoblasts, ANG II in a serum-free medium stimulates early G1 events such as activation of the cyclin-dependent kinase 4 (Cdk4)-cyclin D1 complex, initial hyperphosphorylation of retinoblastoma protein, and partial release of the histone deacetylase 1 inhibitory binding from retinoblastoma protein (28). Interestingly, ANG II also stimulates cyclin E expression in those cells; however, the later G1 phase Cdk2-cyclin E complex was not activated by ANG II stimulation despite the increase in cyclin E protein (28). Consequently, the cells were unable to progress out of the G1 phase after ANG II stimulation alone in this serum-free medium (28). When taken in concert with our present findings that ACE inhibition attenuates myonuclear addition in overloaded soleus muscles, the above in vitro results (28) indicate that ANG II may regulate satellite cell proliferation in vivo by acting synergistically with other factors in overloaded skeletal muscle that control Cdk2-cyclin E activation. One likely candidate is insulin-like growth factor I, which is increased in overloaded skeletal muscle (2) and is known to stimulate Cdk2-cyclin E activation and cell proliferation in cultured primary satellite cells via removal of p27Kip1 inhibition (10).

In this investigation, ACE inhibition attenuated overload-induced muscle hypertrophy in both the plantaris muscle (wet weight, protein content, and fiber cross-sectional area) and the soleus muscle (wet weight), in agreement with a similar previous observation (19). The lack of significant overload-induced increases in protein content or fiber cross-sectional area in the soleus muscle was not necessarily unexpected over this short 7-day time period because increases in muscle protein addition can take many days to reflect increases in muscle wet weight in this overload model (30). Although the lack of overload-induced increases in soleus muscle protein content or fiber cross-sectional area made it impossible to assess the effect of ACE inhibition on these variables, it is highly likely they would reflect the attenuated overload-induced increase in wet weight during a longer overload period. Indeed, it has been demonstrated in two separate experiments that ACE inhibition attenuates soleus muscle hypertrophy as measured by both wet weight and protein content after 10 and 28 days of overload (19). Other observations from the gastrocnemius ablation model have shown that satellite cell proliferation is highest at an early (2–5 day) overload time point (46, 50), whereas large increases in protein content occur later (31). This helps to explain our observed early changes in soleus muscle myonuclear addition in an absence of changes in muscle protein content. Interestingly, ACE inhibition did not alter the overload-induced increase in fast-twitch plantaris muscle myonuclear addition despite attenuating slow-twitch soleus muscle myonuclear addition by ~64%. It is unclear what underlies these contrasting results between muscles, but it is known that inherent satellite cell differences do exist between fiber types, with slow-twitch muscle exhibiting greater satellite cell abundance and rate of proliferation (32, 47). Thus a longer overload period may uncover a potential effect of ACE inhibition on overload-induced plantaris muscle myonuclear addition as well. This possibility is supported by evidence that the myonuclear domain is not as tightly regulated in the fast-twitch plantaris muscle as in the slow-twitch soleus muscle during muscle regrowth (15). Furthermore, differences in soleus vs. plantaris recruitment (24) and subsequent potential differences in loading-induced muscle damage (29) may both play a role in the muscle or fiber-type specific effects of ACE inhibition on overload-induced myonuclear addition.

ES nuclear proliferation was increased with overload in both the soleus and plantaris muscles in this investigation. Zhou et al. (51) also reported an increase in ES nuclei in 1-wk overloaded skeletal muscle but did not attribute this increase to any particular cell type. ANG II has been implicated in fibroblast proliferation in overloaded, hypertrophying cardiac muscle (35). Thus we hypothesized that this phenomenon may exist in skeletal muscle as well, since skeletal muscle fibroblasts can mediate satellite cell proliferation and differentiation (42). Interestingly, our data indicate that ES nuclear proliferation responded similarly to changes in myonuclear addition (i.e., attenuated by ACE inhibition in the soleus but not in the plantaris), indicating that satellite cell activity may be linked to the proliferation of other cell types residing in skeletal muscle.
Surprisingly, although increased with overload, skeletal muscle vimentin-positive areas were not affected by ACE inhibition in this investigation, indicating that the effect of ACE inhibition on ES nuclei in the soleus muscle was not a result of differences in fibroblast content. However, it is possible that the aggregation or size of the fibroblasts was altered by overload or ACE inhibition, which could mask potential changes in fibroblast number using our techniques. Regardless, our finding of an increased vimentin-positive area, along with a qualitative increase in BrdU-positive nuclei within this area in overloaded muscles, extends on the findings of Hansen-Smith et al. (22), who reported fibroblast activation and a qualitative increase in BrdU-positive fibroblast content in 7-day overloaded rat extensor digitorum longus and extensor hallucis proprius muscles. Despite the fact that ANG II stimulates fibroblast proliferation in overloaded hearts (35), our data indicate that ANG II may not play a role in the overload-induced increase in skeletal muscle fibroblast content. Still, it cannot be ruled out that the phenotype of fibroblasts was altered by ACE inhibition in this investigation, leading to secretion of growth factors and extracellular matrix components (8, 21) and thus potentially affecting satellite cell proliferation and/or differentiation (42). It remains to be seen whether ANG II plays a role in altering fibroblast secretion patterns in overloaded skeletal muscle.

Because of the attenuated response in soleus muscle ES nuclear proliferation with ACE inhibition in the absence of a concomitant effect on fibroblast content, it is likely that cell types other than fibroblasts account for this effect of ACE inhibition on ES nuclear proliferation. It is presently unclear exactly which cell types these may be. An obvious possibility is that the attenuated ES nuclear proliferation represents differences in satellite cell proliferation before fusion with existing myofibers. Capillary endothelial cells are another possibility because angiogenesis is known to increase in the compensatory overload model (39) and be attenuated by ACE inhibition (4). Additionally, ACE inhibition may alter immune function (34) and thereby attenuate inflammation and the immune cell infiltration normally observed in overloaded skeletal muscle (5). There is also strong evidence that the proliferation of hemopoietic stem cells, which comprise a small portion of the myogenic precursor nuclei pool (16), is stimulated by ANG II and impeded by ACE inhibition (37). However, it must be noted that hemopoietic stem cells are reported to contribute little to actual mature myonuclear increases in overloaded, hypertrophying skeletal muscle (1).

Although it is possible that a potential effect of ACE inhibition on overload-induced plantaris muscle myonuclear addition was missed by our measurement time point, it is likely that ACE inhibition decreases overload-induced plantaris muscle hypertrophy at least partly by affecting mechanisms other than myonuclear addition. For instance, ACE inhibition may suppress protein synthesis because ANG II is known to mediate loading-induced protein synthesis in vascular smooth muscle cells and cardiac myocytes through ANG II type 1 (AT₁) receptor activation (7). Interestingly, Gordon et al. (19) demonstrated that optimal overload-induced skeletal muscle hypertrophy is dependent on the AT₁ receptor, which is located on mature muscle fibers and in the skeletal muscle vasculature (33). In this respect, Carson et al. (9) found that 3 days of plantaris muscle overload after gastrocnemius ablation results in a 7.2-fold increase in AT₁ receptor gene expression. Such results also raise the possibility that satellite cell proliferation itself is mediated via the AT₁ receptor or that there could be fiber-type differences in AT₁ receptor density. Although it is known that smooth muscle cell and hemopoietic stem cell proliferation are mediated by the AT₁ receptor (37, 40), this possibility remains to be explored in skeletal muscle satellite cells.

In summary, ACE inhibition in rats significantly attenuated myonuclear addition and ES nuclear proliferation in 7-day overloaded slow-twitch soleus muscles but not overloaded fast-twitch plantaris muscles. Skeletal muscle overload also led to an increase in fibroblast content in both muscles that was unaffected by ACE inhibition. These findings indicate that ANG II may mediate myonuclear addition in overloaded slow-twitch muscle but independently of changes in fibroblast content. Furthermore, ANG II may not mediate myonuclear addition in overloaded fast-twitch muscle, at least during the first 7 days of the overloading stimulus. It is presently unclear why there are muscle- or fiber-type-specific effects of ACE inhibition on overload-induced satellite cell activity. Nevertheless, our results provide further insight into the mechanisms underlying the involvement of ANG II in overload-induced skeletal muscle hypertrophy (19).

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