In vivo inhibition of nitric oxide synthase impairs upregulation of contractile protein mRNA in overloaded plantaris muscle

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Sellman, Jeff E., Keith C. DeRuisseaux, Jenna L. Betters, Vitor A. Lira, Quinlyn A. Soltow, Joshua T. Selsby, and David S. Criswell. In vivo inhibition of nitric oxide synthase impairs upregulation of contractile protein mRNA in overloaded plantaris muscle. J Appl Physiol 100: 258–265, 2006. First published September 15, 2005; doi:10.1152/japplphysiol.00936.2005.—Inhibition of nitric oxide synthase (NOS) activity in vivo impedes hypertrophy in the overloaded rat plantaris. We investigated the mechanism for this effect by examining early events leading to muscle growth following 5 or 12 days of functional overload. Male Sprague-Dawley rats (~350 g) were randomly divided into three treatment groups: control, Nω-nitro-arginine methyl ester (L-NAME; 90 mg·kg⁻¹·day⁻¹), and L-arginine (L-Arg; 90 mg·kg⁻¹·day⁻¹). Unilateral removal of synergists induced chronic overload (OL) of the right plantaris. Sham surgery performed on the left hindlimb served as a normally loaded control. L-NAME and TRIM treatments prevented OL-induced skeletal α-actin and type I (slow) myosin heavy chain mRNA expression at 5 days. Conversely, neither L-NAME nor TRIM affected hepatocyte growth factor or VEGF mRNA responses to OL at 5 days. However, OL induction of IGF-I and mechanogrowth factor mRNA was greater (P < 0.05) in the TRIM group compared with the controls. Furthermore, the phosphorylated-to-total p70 S6 kinase ratio was higher in OL muscle from NOS-inhibited groups, compared with control OL. At 12 days of OL, the cumulative proliferation of plantaris satellite cells was assessed by subcutaneous implantation of time release 5-bromo-2′-deoxyuridine pellets during the OL-inducing surgeries. Although OL caused a fivefold increase in the number of mitotically active (5-bromo-2′-deoxyuridine positive) sublaminar nuclei, this was unaffected by concurrent NOS inhibition. Therefore, NOS activity may provide negative feedback control of IGF-I/p70 S6 kinase signaling during muscle growth. Moreover, NOS activity may be involved in transcriptional regulation of skeletal α-actin and type I (slow) myosin heavy chain during functional overload.

ADULT SKELETAL MUSCLE IS EXTREMELY adaptable to changes in mechanical loading. Chronic overload causes structural and phenotypic alterations in muscle fibers, resulting in dramatic muscle growth. This hypertrophy response involves the activation and subsequent fusion of satellite cells to the muscle fibers (30, 31) and increased synthesis of structural and contractile proteins (18, 40). Concomitantly, capillary angiogenesis supports the growing muscle (29). The molecular regulation of the growth factors that govern this complex adaptation in skeletal muscle is poorly understood.

IGF-I is of prime importance for maintenance and growth of adult skeletal muscle mass (1). IGF-I is a potent stimulator of anabolic, mitogenic, and myogenic processes in skeletal muscle (27, 38). While circulating IGF-I can certainly affect skeletal muscle, it is now clear that autocrine/paracrine actions of endogenously produced IGF-I are critical for muscle hypertrophy (1, 3, 9). In fact, Goldspink and colleagues (25, 41) have identified and cloned a splice variant of IGF-I, called mechanogrowth factor (MGF), that is particularly responsive to loading and/or injury in adult skeletal muscle. IGF-I activates Akt/mammalian target of rapamycin-dependent phosphorylation of p70 S6 kinase (p70S6K) with subsequent increase in protein translation (5). A direct link between nitric oxide (NO) signaling and skeletal muscle IGF-I expression has not been established. However, dystrophic muscle, in which neuronal NO synthase (nNOS) protein is not associated with the sarcolemma and myotendinous junction (7), fails to express IGF-I in response to stretch or overload (17). This suggests the possibility of a link between NOS activity and IGF-I induction.

Skeletal muscle hypertrophy and fiber-type adaptations during overload are also supported by increased transcriptional activity, as evidenced by the increase in skeletal α-actin and type I (slow) myosin heavy chain (MHC) mRNA and promoter activity (6, 16). Nevertheless, the signal(s) linking mechanical load to skeletal myofiber IGF-I gene expression, p70S6K activation, and transcriptional upregulation remains a mystery.

Satellite cell activation and proliferation are also required for adult muscle growth (28). The activation of quiescent satellite cells is governed by hepatocyte growth factor (HGF). HGF is sequestered to the extracellular matrix in adult, uninjured skeletal muscle (32, 36). Muscle stretch or injury releases HGF via a NO-dependent mechanism (4) to bind to the c-met receptor on quiescent satellite cells. Although sera from animals undergoing muscle regeneration can induce HGF expression (34), the specific factors regulating transcription of this growth factor during muscle overload are unknown.

Finally, skeletal muscle hypertrophy is closely linked to blood vessel recruitment and capillary angiogenesis, such that capillary density is maintained during the early stages of hypertrophy (29). VEGF is the primary regulator of angiogenesis and is highly induced in growing and hypertrophying skeletal muscle (35). Recently, expression of VEGF in skeletal muscle cells has been shown to be NOS dependent (15) and under the control of IGF-I-dependent Akt signaling (35).

The exquisite coordination of this complex response to skeletal muscle overload suggests a common regulator. We have reported that inhibition of NOS activity interferes with the normal hypertrophy and fiber-type adaptations to chronic skel-
et al muscle overload (33). In this study, we examined the sensitivity to NOS inhibition of several key components of the hypertrophy response in the rat plantaris. Specifically, we measured growth factor expression (IGF-I, MGF, HGF, VEGF), activation of contractile protein transcription (skeletal α-actin and type I MHC mRNA), activation of protein translation (p70S6K phosphorylation), and satellite cell proliferation [cumulative 5-bromo-2-deoxyuridine (BrDU) labeling and histocchemistry] following 5 or 12 days of functional overload. We hypothesized that inhibition of NOS activity would prevent responses of these pathways to chronic overload.

NOS inhibition during plantaris overload did not attenuate growth factor transcript expression or the amount of mitotically active satellite cells. Indeed, nNOS-specific inhibition further increased IGF-I and MGF mRNA during overload, suggesting the possibility of NO-dependent feedback control of IGF-I signaling and/or regulation of mRNA stability. Consistent with the elevated IGF-I transcripts, the phosphorylation status of p70S6K was significantly higher in OL muscle from N0-t-nitro-l-arginine methyl ester (l-NAME) and l-(2-trifluoromethylphenyl)-imidazole (TRIM) groups, compared with control. The data indicate that the negative effects of NOS inhibition on skeletal muscle hypertrophy are not mediated via effects on growth factor induction or satellite cell activation/proliferation. On the other hand, upregulation of actin and type I MHC mRNA was prevented by NOS inhibition. Therefore, endogenous NO production may be involved in the upregulation of contractile protein mRNA during overload-induced hypertrophy.

METHODS

Animals. Male Sprague-Dawley rats (6 mo old, ~350 g) were purchased from Harlan (Indianapolis, IN). Rats were individually housed in polycarbonate cages in the University of Florida Health Science Center Animal Facility on a 12:12-h light-dark cycle (light 0700–1900). The University of Florida Institutional Animal Care and Use Committee approved the protocol for this study.

Systemic inhibition of NOS activity. The pharmacological inhibition of NOS was achieved by administering the competitive nonspecific NO donor l-NAME (Sigma Chemical) or the nNOS-selective inhibitor TRIM (Cayman Chemical) during the treatment period. Rats were randomly divided among control, l-NAME, and TRIM groups (n = 8 rats/group for experiment 1, and control and l-NAME (n = 4/group) for experiment 2. l-NAME was given in the rats’ drinking water at a concentration of 0.75 mg/ml, producing a dose of ~90 mg·kg body mass⁻¹·day⁻¹; a dose previously shown to effectively inhibit NO synthesis and skeletal muscle NOS activity (33). TRIM was dissolved in PBS and injected intraperitoneally once daily at a dose of 10 mg·kg⁻¹·day⁻¹. To control for potential injection effects, control and l-NAME rats were injected daily with a volume of PBS equal to the volume injected into the TRIM animals. TRIM has significant inhibitory effects on both nNOS and inducible NOS (iNOS). nNOS and endothelial NOS (eNOS) are constitutively expressed in skeletal muscle (24), but iNOS is not. Although we confirmed by immunoblot that iNOS protein was not expressed in 5-day normally loaded (NL) or overloaded (OL) plantaris muscles (data not shown), iNOS could have been transiently expressed during the OL period. Therefore, TRIM effects could be due to nNOS and/or iNOS inhibition.

Synergist ablation surgery. Chronic overload of the plantaris was induced by surgical, unilateral removal of the synergist muscles to the plantaris. The rats were anesthetized with inhaled isoflurane (2–5%) with oxygen as the carrier gas. By using aseptic technique, a midline incision was made in the skin of the right hindlimb, from the popliteal fossa to the Achilles tendon region. A second longitudinal incision was made through the hamstrings, exposing the distal gastrocnemius and Achilles tendon region. The gastrocnemius tendon was carefully separated from the plantaris tendon, and the gastrocnemius muscle was sectioned. The distal two-thirds of the gastrocnemius was removed, taking care not to disturb the plantaris nerve and blood supply. Next, the soleus muscle was carefully isolated and removed. The hamstring incision was closed with 4-0 vicryl absorbable suture. The overlying skin was closed with sterilized metal wound clips and treated with a topical antibiotic cream to avoid infection.

A sham operation was performed on the left leg of each rat. In this procedure, the same incisions were made, and the gastrocnemius tendons isolated, but without transecting the muscles. The animals were allowed to fully recover from the anesthetic before returning to their cages. The rats were examined daily for signs of infections or wound openings, which were promptly treated, if found. If the animals favored the sham-operated limbs (nonablated), the NL muscles could have been minimally loaded above baseline. This would underesti- mate the OL effect. Nevertheless, the plantaris mass-to-body mass ratio, the degree of hypertrophy, and the l-NAME effects on hyper trophy using this unilateral ablation model were very similar to our laboratory’s previously reported results using a bilateral ablation model (33).

Experimental protocol. To ensure effective inhibition of NOS activity at the onset of muscle overload, l-NAME was added to the drinking water of the appropriate group (l-NAME), and daily injec- tions of TRIM (TRIM group) or PBS (control and l-NAME groups) were begun 2 days before the animals underwent surgery. Forty-eight hours after beginning the l-NAME and TRIM treatments, all rats received the unilateral synergistic ablation and sham surgeries. After surgery, the rats were housed individually and allowed to ambulate freely in the cage for 5 days. Drinking water was replaced, and body mass was recorded daily throughout the experimental period, and the dose of l-NAME, per kilogram of body mass, was calculated for each l-NAME rat. In addition, the rats were examined to ensure proper healing of the wounds. On day 5 postsurgery, the rats were anesthetized with isoflurane and killed by exsanguination. Immediately after the rats were killed, the plantaris muscles were removed, trimmed of connective tissue and fat, weighed on an analytical balance, frozen in liquid nitrogen at resting length, and then stored at −80°C for subsequent biochemical analyses.

A second experiment was conducted to cumulatively label mitotically active cells during the functional overload treatment. Control and l-NAME groups were treated, and the plantaris muscle was unilaterally overloaded as described above. In these animals, BrdU constant-release pellets (4 mg BrdU/day; Innovative Research of America, Sarasota, FL) were implanted subcutaneously during the ablation surgeries using aseptic technique via a small incision above the scapulae. The pellet remained in place until the animal was killed, at which point the pellet was removed and weighed to confirm release of the appropriate quantity of BrdU. Following 12 days of functional overload, plantaris muscles were harvested as described above and frozen at resting excised length by submersion in isopentane, cooled to the temperature of liquid nitrogen. Frozen samples were stored at −80°C for subsequent immunohistochemical analyses.

Serum nitrate/nitrite. Systemic NOS inhibition was confirmed by measurement of total nitrate plus nitrite in serum samples from each animal. Whole blood collected at the time of death was allowed to coagulate on ice followed by centrifugation at 2,000 g for 10 min. Serum was collected and frozen (−80°C) for subsequent analysis. Samples were thawed, diluted 1:1 with PBS, and filtered with Milli- pore UltrafreeMC microcentrifuge filter cups (10,000 molecular weight cutoff). Aliquots from each sample were then analyzed in triplicate by using a fluorometric assay kit for nitrate plus nitrite (Cayman Chemical).

Reverse transcription and real-time PCR. Total RNA was isolated from muscle tissue using TRIzol Reagent (Life Technologies, Carls-

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bad, CA), according to the manufacturer’s instructions. The amount of total RNA was evaluated by spectrophotometry, and the integrity was checked by gel electrophoresis. Total RNA (5 μg) was reverse transcribed using the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) using oligo(dT)20 primers and the protocol outlined by the manufacturer. Samples were run in triplicate. One microliter of cDNA (5 μl for MGF) was added to a 25-μl PCR reaction for real-time PCR using Taqman chemistry and the ABI Prism 7000 Sequence Detection System (ABI, Foster City, CA). Relative quantitation of gene expression was performed using the comparative computed tomography method (ABI, User Bulletin no. 2) using hypoxanthine guanine phosphoribosyl transferase (HPRT) as the normalizer. HPRT was chosen as the reference gene based on initial experiments showing unchanged expression with our experimental manipulations. Fivefold dilution curves were assayed on selected samples to confirm the validity of this quantitation method for each gene. IGF-I (GenBank NM_178866), VEGF (GenBank NM_031836), type I (slow) MHC (GenBank NM_017250), and skeletal α-actin (GenBank NM_019212) mRNA transcripts were assayed using predesigned rat primer and probe sequences commercially available from Applied Biosystems (Assays-on-Demand). MGF [5′-CCTGCAGATGCGCAAGACTCA (forward) and 5′-ATTGGCCGTCTCCAGCTTG (reverse)] and HPRT [5′-GTTGGAATACGCGACTTTTG (forward) and 5′-AGTCAAGGGCATATCCAACAACAA (reverse)] mRNA were assayed using custom-made primers (Applied Biosystems, Assays-by-Design). The MGF reverse primer was custom designed to span the 52-base pair insert unique to the rat MGF cDNA.

Semiquantitative RT-PCR. Total RNA was reverse transcribed using Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences, Buckinghamshire, UK) and a combination of random hexamers and oligo(dT)20 primers. cDNA for the four isoforms of VEGF were synthesized using Ready-to-Go You-Prime First-Strand Beads (Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer’s instructions, and protein levels were determined by densitometry (Scion Image software). Blots were stripped (Restore Stripping Buffer, Pierce Chemical) and re-probed with primary antibody for total p70S6K (Cell Signaling Technology), then stripped again and probed for β-actin (Abcam) for control for loading errors.

Immunohistochemical identification of proliferating satellite cells and myonuclei. Transverse sections of the frozen plantaris muscles were cut at −20°C and mounted serially on adherent-coated slides for subsequent staining of proliferating nuclei, total nuclei, and basal lamina. Slide sections were fixed in 3.7% formaldehyde for 15 min and incubated in 2 M HCl for 20 min at 37°C. Muscle sections were blocked in 5% goat serum for 1 h at RT. Incubation with primary antibodies, anti-BrdU-FITC (Roche), and anti-laminin (Sigma, St. Louis, MO) was performed for 1 h at 37°C with 1:20 and 1:25 dilution, respectively. After washing, incubation with a Texas red-conjugated anti-rabbit secondary antibody was carried out for 1 h at 37°C. The sections were washed again and mounted in medium containing 0.1% 4′,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories). A Zeiss Axiopt fluorescence microscope coupled to a digital camera was used to acquire images. All myonuclei (DAPI positive) and all mitotically active (DAPI and BrdU positive) myonuclei lying within the laminin boundary were counted within three separate fields of view taken randomly from superficial, intermediate, and deep portions of each muscle section (×40 magnification; 400–600 total myonuclei per muscle). The sum of myonuclei and BrdU-positive nuclei from the three fields of view were used to calculate a ratio of BrdU-positive nuclei per 1,000 myonuclei for each muscle.

Statistical analyses. Main effects and interactions for each variable were analyzed by using a two-way ANOVA (treatment × loading condition) with repeated measures on the loading condition. Tukey’s honestly significant difference test was applied post hoc to determine individual group differences. Significance was established a priori at P < 0.05.

RESULTS

Body mass did not change from pre- to post-OL treatment for any group. Furthermore, mean body mass (±SE) did not differ between groups at any time point (Table 1). Consistent with the report of Adams and Haddad (2), plantaris total protein was not significantly different between NL and OL muscles at 5 days. However, in the 12-day-treated animals, plantaris total protein was elevated 45% in the control OL group compared with control NL (Table 1). L-NAME treatment inhibited the accumulation of protein in the 12-day animals by ∼50% (Table 1). Water consumption in the L-NAME-treated

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Mass, g</th>
<th>Loading</th>
<th>Plantaris Mass, mg</th>
<th>Total Protein, mg</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>5-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>340.4 ± 8.9</td>
<td>NL</td>
<td>323.0 ± 21.1</td>
<td>60.8 ± 5.8</td>
</tr>
<tr>
<td>L-NAME</td>
<td>8</td>
<td>334.4 ± 11.5</td>
<td>OL</td>
<td>430.8 ± 13.9</td>
<td>64.1 ± 3.9</td>
</tr>
<tr>
<td>TRIM</td>
<td>8</td>
<td>340.1 ± 9.8</td>
<td>NL</td>
<td>342.5 ± 19.3</td>
<td>62.1 ± 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OL</td>
<td>385.0 ± 20.4</td>
<td>65.3 ± 6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>353.4 ± 24.1</td>
<td>NL</td>
<td>374.2 ± 31.1</td>
<td>71.2 ± 5.7</td>
</tr>
<tr>
<td>L-NAME</td>
<td>4</td>
<td>384.9 ± 18.2</td>
<td>OL</td>
<td>624.9 ± 37.2</td>
<td>103.3 ± 7.1†</td>
</tr>
<tr>
<td>TRIM</td>
<td>4</td>
<td>366.1 ± 21.5</td>
<td>NL</td>
<td>366.1 ± 21.5</td>
<td>74.1 ± 5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OL</td>
<td>477.6 ± 33.4†</td>
<td>89.8 ± 5.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. L-NAME, Nω-nitro-L-arginine methyl ester; TRIM, 1-(2-trifluoromethyl-phenyl)-imidazole; NL, contralateral normally loaded; OL, overloaded. Significantly different from *corresponding NL (within group), and †control OL: P < 0.05.
Skeletal α-actin and type I (slow) MHC mRNA expression.

Five days of OL resulted in a 90% increase in skeletal α-actin mRNA and a 140% increase in type I (slow) MHC mRNA (Fig. 1; control NL vs. control OL). However, the data indicate a complete inhibition of this OL-induced response in the NOS-inhibited groups. Skeletal α-actin and type I (slow) MHC mRNA levels did not differ between NL and OL muscles in either treatment group (L-NAME and TRIM). Furthermore, α-actin and type I (slow) MHC transcript levels in L-NAME and TRIM muscles did not differ from that in control NL muscles.

Growth factor mRNA expression. Expression of mRNA for HGF, IGF-I, MGF, and the 120-amino acid splice variant of VEGF (VEGF-120) were all increased in the OL muscles. IGF-I mRNA was increased approximately fourfold and MGF mRNA approximately ninefold in control OL compared with control NL muscles. TRIM treatment did not affect IGF-I or MGF mRNA expression in the NL muscle, but it approximately doubled expression of both transcripts in the OL muscle compared with control OL (Table 2). HGF and VEGF-120 mRNAs were increased 15- to 20-fold in the OL muscles compared with NL, with no effect of either L-NAME or TRIM treatment (Table 2 and Fig. 2). Transcripts for VEGF-188, VEGF-164, and VEGF-144 tended to be reduced in the OL muscles, but this did not reach statistical significance (P > 0.05). Real-time PCR assessment of total VEGF mRNA showed an ~50% reduction in OL muscle, compared with NL. The discrepancy between real-time assessment of total VEGF mRNA (Table 2) and semiquantitative assessment of VEGF isoform expression (Fig. 2) is most likely due to the relatively small contribution of VEGF-120 to the total VEGF mRNA pool and the variability in the semiquantitative RT-PCR method, which failed to demonstrate a significant OL-related reduction in the more abundant VEGF isoforms.

Phosphorylation of p70S6K. Expression of total p70S6K protein was increased in OL muscles in all three treatment groups. Similarly, phosphorylated p70S6K and the ratio of phosphorylated to total p70S6K were increased in OL muscles compared with NL. Compared with control OL muscles, however, the ratio of phosphor to total p70S6K was significantly higher in OL muscles from L-NAME and TRIM animals, indicating a greater relative phosphorylation status (Fig. 3). β-Actin protein did not differ between any of the groups.

Satellite cell activation/proliferation. The number of BrdU-positive myonuclei identified in histological sections of the OL plantaris muscles was approximately fourfold higher than in

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**Table 2. Real-time RT-PCR quantification of mRNA transcripts for selected growth factors in the plantaris muscle following 5 days of OL.**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Control (NL)</th>
<th>Control (OL)</th>
<th>L-NAME (NL)</th>
<th>L-NAME (OL)</th>
<th>TRIM (NL)</th>
<th>TRIM (OL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>1.12±0.18</td>
<td>3.98±0.28*</td>
<td>1.06±0.18</td>
<td>5.06±0.59†</td>
<td>1.22±0.14</td>
<td>8.49±0.86†‡</td>
</tr>
<tr>
<td>MGF</td>
<td>1.07±0.33</td>
<td>8.84±0.78*</td>
<td>0.73±0.12</td>
<td>11.32±3.05‡</td>
<td>0.99±0.29</td>
<td>22.03±6.31†‡</td>
</tr>
<tr>
<td>HGF</td>
<td>1.18±0.30</td>
<td>19.76±4.1*</td>
<td>2.45±0.80</td>
<td>20.78±2.7*‡</td>
<td>1.85±0.39</td>
<td>17.78±0.62†‡</td>
</tr>
<tr>
<td>Total VEGF</td>
<td>1.22±0.27</td>
<td>0.58±0.09*</td>
<td>1.68±0.52</td>
<td>0.68±0.14†</td>
<td>1.94±0.50</td>
<td>0.52±0.08†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. Transcripts are normalized to hypoxanthine guanine phosphoribosyl transferase mRNA and expressed relative to the control NL value, using the comparative computed tomography method. MGF, mechanogrowth factor; HGF, hepatocyte growth factor. Significantly different from *control NL, †corresponding NL (within group), and ‡control OL: P < 0.05.
However, L-NAME treatment had no effect on the number of BrdU-positive myonuclei (Fig. 4).

DISCUSSION

To our knowledge, this is the first study to investigate the effects of functional overload on the upregulation of key signaling pathways, leading to increased protein synthesis, angiogenesis, and satellite cell activation and proliferation. Our primary observations were focused on an early time point (5 days of overload) when growth factor expression is at its peak (2). Although protein accumulation is not elevated at this point (Ref. 2; Table 1), events leading to muscle hypertrophy are induced, including growth factor expression, contractile gene transcription, and protein synthesis rate (2, 25). The data support our hypothesis that NOS activity is important for upregulation of contractile gene expression (actin and slow MHC mRNA). Conversely, neither L-NAME nor TRIM treatment repressed the overload-related increase in skeletal muscle growth factor mRNA expression. In fact, the TRIM OL group expressed approximately double the MGF and IGF-I transcripts compared with control OL, which are remarkably similar to the L-NAME effects. As TRIM inhibits iNOS as well as nNOS, our data support a role for one or both of these NOS isoforms in early adaptations to skeletal muscle overload.

Skeletal α-actin and type I (slow) MHC mRNA expression. Skeletal α-actin protein is an important component of the contractile apparatus and is known to be transcriptionally upregulated during skeletal muscle hypertrophy (6). Because adult skeletal muscle sarcomeric actin is derived from the single α-skeletal actin gene, rather than from multiple iso-

the NL muscles (12 days of OL). However, L-NAME treatment had no effect on the number of BrdU-positive myonuclei (Fig. 4).

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Employing in vivo models to investigate NO signaling, although physiologically meaningful, is not without consequence. Systemic nonisoform-specific NOS inhibition (L-NAME) has physiological consequences throughout the body and can lead to significant effects on hemodynamics (8), muscle contractility (13), and gene expression in nonmuscle tissue (21). The majority of these systemic L-NAME effects are thought to be secondary to eNOS inhibition and the resulting effects on blood flow and blood pressure. In fact, the nNOS-specific inhibitor TRIM has been administered to rats in vivo with no reported systemic side effects (24). Since nNOS is the most abundant NOS isoform in skeletal muscle (37) and is reportedly sensitive to muscle loading (37), we hypothesized that this isoform accounts for overload-induced NO signaling in the plantaris muscle. To test this possibility, and partially control for the systemic effects of L-NAME, we treated one group of rats with daily intraperitoneal injections of TRIM. Although we have not confirmed that TRIM treatment inhibits OL-induced protein accumulation, the effects of TRIM on muscle growth-related events at 5 days of OL are remarkably similar to the L-NAME effects. As TRIM inhibits iNOS as well as nNOS, our data support a role for one or both of these NOS isoforms in early adaptations to skeletal muscle overload.

Skeletal α-actin and type I (slow) MHC mRNA expression. Skeletal α-actin protein is an important component of the contractile apparatus and is known to be transcriptionally upregulated during skeletal muscle hypertrophy (6). Because adult skeletal muscle sarcomeric actin is derived from the single α-skeletal actin gene, rather than from multiple iso-

Fig. 2. Semiquantitative RT-PCR analysis of VEGF mRNA splice variant expression. A: mean ± SE of mRNA expression levels of VEGF-120, relative to ribosomal 18S RNA, in 5-day OL and NL plantaris muscles of control, L-NAME-, and TRIM-treated rats. B: representative ethidium bromide-stained 1% agarose gel illustrating PCR products following amplification of VEGF and 18S. See METHODS for details of assay conditions. *Significantly different from corresponding NL, P < 0.05.

Fig. 3. A: representative immunoblot for phosphor(Thr^389)-p70 S6 kinase (p70^S6K), total-p70^S6K, and β-actin (loading control) in 5-day OL and NL plantaris muscles of control, L-NAME-, and TRIM-treated rats. B: quantification phosphor(Thr^389)-p70^S6K to total p70^S6K ratio. Values are means ± SE, expressed relative to control NL mean. *Significantly different from corresponding NL, P < 0.05. **Significantly different from control OL, P < 0.05.
forms, the regulation of this gene serves as an index of overall contractile protein synthesis. MHC, on the other hand, can be produced from at least four different genes in adult skeletal muscle and is an important determinant of fiber-type-specific characteristics. Carson et al. (6) have reported that transcriptional activity of the actin promoter is increased in skeletal muscle during in vivo stretch overload. This effect is mediated by serum response factor and transcriptional enhancer factor-1 binding to the actin promoter (6). Meanwhile, Giger et al. (16) report that functional overload of the rat plantaris results in transcriptional activation of the type I (slow) MHC gene and that this response is mediated via a putative transcriptional enhancer binding element. A paper (22) found that NO donors were sufficient to induce serum response factor binding to a MHC promoter element and increase promoter activity in cultured smooth muscle cells. Our data suggest that nNOS activity is important for induction of transcription of these genes during chronic overload, but further study will be required to determine the mechanism of this effect.

**HGF expression and satellite cell activity.** Release of HGF and subsequent mobilization and activation of resident satellite cells in skeletal muscle, in vitro, are dependent on NO signaling (4). Nevertheless, our in vivo data do not support the idea that NO is necessary for overload induction of HGF expression or the activation/proliferation of satellite cells. Although muscle satellite cells become active during the first week of syner gent ablation OL (28), the rate of increase in muscle mass and total protein peaks between 10 and 15 days of OL (2). We chose to measure sublaminar BrdU-positive nuclei following 12 days of OL to get a cumulative assessment of overall satellite cell activation and proliferation. Still, we cannot fully rule out the possibility of an L-NAME effect on satellite cell activity, since some activated satellite cells could have been lost by apoptosis during the 12-day period. Furthermore, our histochemical methods did not determine the proportion of activated cells that had fused with the sarcolemma. Therefore, it is possible that NOS inhibition could interfere with hypertrophy by affecting cell fusion.

Another limitation of our methods is the assumption that sublaminar nuclei represent only myonuclei or satellite cells, which is not confirmed by muscle/satellite cell-specific markers. Therefore, our interpretation must be restricted to the conclusion that cumulative OL-induced cellular proliferation was unaffected by NOS inhibition.

**VEGF expression.** The reduction in total VEGF mRNA level in the 5-day OL muscle was unexpected, given the known capillary angiogenesis occurring in OL rat muscle (29). However, the few studies reporting VEGF mRNA expression in hypertrophying skeletal muscle show mixed results. Degens et al. (11) found no significant changes in VEGF mRNA in hypertrophying quail muscle. Similarly, 4 wk of strength training in human subjects, even under hypoxic conditions, did not change skeletal muscle VEGF mRNA expression (14). On the other hand, overload of the rat plantaris for 2 wk (i.e., 2.8× longer than our 5-day treatment) did increase VEGF mRNA by ~50% (12).

To further characterize the VEGF mRNA response, we examined expression of the four splice-variant isoforms found in rat skeletal muscle (10) using semiquantitative RT-PCR and published primer sequences (10). To our knowledge, ours is the first study to measure expression of specific VEGF mRNA splice variants in OL, hypertrophying skeletal muscle. Unlike aerobic exercise, which primarily induces the VEGF-164/5 isoform (19, 23), we found that the VEGF-120 isoform was induced in the 5-day OL plantaris muscle. This effect, however, was not influenced by L-NAME or TRIM treatments. The differential effects of the VEGF isoforms are unclear. Nevertheless, gene transfer of human VEGF-120 into rat skeletal muscle confirms that this isoform induces angiogenesis (26).

**IGF-I expression and phosphorylation of p70S6K.** These results are consistent with previous findings that endogenous IGF-I mediates adult skeletal muscle hypertrophy (1, 3), as our data show a dramatic increase in the amount of transcript with OL. Additionally, we show that the MGF splice variant is particularly responsive to overload. These data support a role for NO that is either independent of the IGF-I axis or downstream of IGF-I transcription. The exaggerated expression of IGF-I and MGF mRNA in the OL plantaris of the nNOS-inhibited TRIM group suggests the possibility of a nNOS-dependent negative feedback or mRNA stability mechanism controlling the load-induced IGF-I response, IGF-I/Akt signaling in the rat kidney is known to activate eNOS by phosphorylation and increase NO production (39). The phosphorylation status of the NOS isoforms in OL skeletal muscle is unknown, but it seems possible that IGF-I-dependent nNOS activation could produce a feedback signal to control IGF-I/MGF mRNA expression during muscle growth.

Fig. 4. Cumulative 5′-bromo-2′-deoxyuridine (BrdU) labeling of mitotically active cells in the plantaris during 12 days of chronic overload. A: representative histological section showing immunofluorescent identification of laminin (red) and BrdU (green), with 4′,6-diamidino-2-phenylindole dihydrochloride counterstain (blue) to identify nuclei. BrdU-positive nuclei lying within the basal lamina are identified with arrows. B: mean ± SE of BrdU-positive myonuclei (sublaminar) per 1,000 myonuclei. *Significantly different from corresponding NL, P < 0.05.
Activation of the key translational regulator, p70S6K, is correlated to increased protein synthesis in skeletal muscle, induced by phosphoinositide 3-kinase or mechanical stretch (20). As NOS inhibition reduces protein accumulation in OL rat plantaris at 12 days, we postulated that phosphorylation of p70S6K at 5 days would be inhibited in the t-NAME and TRIM groups. On the contrary, we found that OL induced expression of total p70S6K protein in all groups and that this corresponded to an increase in phosphorylated p70S6K in the OL muscles. However, the ratio of phosphor to total p70S6K, indicating the relative degree of activation of the pathway, was significantly higher in the OL muscles of the t-NAME and TRIM groups. This suggests that protein translation could have been elevated in partial compensation to reduced transcriptional activity. Further experiments are needed to directly measure effects of NO on skeletal muscle protein synthesis rates during hypertrophy.

In conclusion, upregulation of skeletal α-actin and type I (slow) MHC mRNA (presumably via transcription) during chronic skeletal muscle overload is dependent on nNOS activity. Conversely, induction of growth factors, activation of protein translation (p70S6K phosphorylation), and satellite cell activation/proliferation are not dependent on NOS activity. Nevertheless, NO production may provide feedback control of IGF-I and MGF signaling in hypertrophying muscle.

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REFERENCES


