Eccentric exercise induces nitric oxide synthase expression through nuclear factor-κB modulation in rat skeletal muscle

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Lima-Cabello E, Cuevas MJ, Garatachea N, Baldini M, Almar M, González-Gallego J. Eccentric exercise induces nitric oxide synthase expression through nuclear factor-κB modulation in rat skeletal muscle. J Appl Physiol 108: 575–583, 2010. First published December 31, 2009; doi:10.1152/japplphysiol.00816.2009.—This study aimed to investigate the effect of eccentric exercise on the expression of different nitric oxide synthase (NOS) isoforms in rat deep vastus lateralis muscle. Twenty-four rats were allocated to four experimental groups: rested control group, acutely exercised group after an intermittent downhill protocol for 90 min, acutely exercised group treated with pyrrolidine dithiocarbamate (100 mg/kg ip) for 24 and 1 h before the acute exercise bout, and acutely exercised group with previous submaximal eccentric training of 8 wk. Acutely exercised rats showed increased levels of protein tyrosine nitration, NF-κB binding, and phospho-IκBα content. A significant increase was observed in mRNA level and protein content of neuronal NOS, inducible NOS, and endothelial NOS. The binding of NF-κB to the NOS isoform promoters, measured by a chromatin immunoprecipitation assay, was undetectable in rested rats, whereas it was evident in acutely exercised animals. All of these effects were partially abolished by pyrrolidine dithiocarbamate treatment and by training. In summary, our findings provide a direct link between the NF-κB signaling cascade and NOS expression in skeletal muscle following eccentric exercise and suggest a modulation of the expression of the three NOS isoforms by this transcription factor.

Nitric oxide (NO), a molecule with diverse physiological functions, is produced in vivo through the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). The family of NOS enzyme consists of three members: type I, neuronal NOS (nNOS); type II, inducible NOS (iNOS); and type III, endothelial NOS (eNOS). These isoforms, although structurally related, differ from each other in their genetic origin, anatomic distribution, ion dependence for activity, and pathophysiological functions (52). NO is generated continuously by skeletal muscle, a production that is increased by a variety of chemical and physical factors, raising the possibility that ROS represent common signaling molecules for modulating eNOS expression (20). Increasing evidence indicates that NOS expression can also be dynamically regulated by various physiological conditions (33–34), and it is known that nNOS and eNOS expression increases in rat limb muscles after chronic exercise or intense short-term exercise training (37, 51). Transcription factors involved in activation of the genes encoding for eNOS and NOS have not been unequivocally identified, but NF-κB is an attractive candidate, since those genes contain putative binding sites for NF-κB in their promoter region (12, 19, 48). Although it has been reported that acute exercise may be associated with the induction of iNOS and eNOS in skeletal muscle (2, 17), a cause-effect link between NF-κB activation and changes in the expression of NOS isoforms has not been demonstrated. Because unaccustomed eccentric exercise is clearly documented to induce an inflammatory response accompanied by marked NO overproduction (10, 39), in this study we examined the effect of an acute bout of eccentric exercise on the expression of the different isoforms of NOS in rat skeletal muscle. To gain some.
insight into the mechanisms responsible for the exercise-induced changes in enzyme expression, the effect of pyrrolidine dithiocarbamate (PDTC), a well-known inhibitor of NF-κB used widely as a tool in the study of the regulation of gene expression, was determined. Because physical training has been associated with a decrease in markers of inflammation and iNOS expression induced by acute exercise (27), we also investigated the potential effects of eccentric training on the changes in expression of NOS isoforms and NF-κB activation induced by acute exercise.

MATERIALS AND METHODS

Animals. All experiments were approved by the Institutional Animal Care Committee of the University of León and conform to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Male Wistar rats weighing 200 ± 10 g were used in all experiments. The animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12:12-h light-dark cycle.

Experimental design. All animals were initially acclimated to running on a motor-driven treadmill designed for rats (model LI8706; Letica, Barcelona, Spain), beginning at 8 min/min for 10 min the week previous to the exercise performance. This protocol accustomed the rats to the locomotion involved in the final exercise experiments without resulting in training adaptation in skeletal muscle. Thirty-two animals were randomly divided into four groups. The first group (control) was injected with 1 ml of saline and rested for 2 h before killing. A second group of rats performed an acute bout of exercise, which consisted of an intermittent downhill protocol (3) with incline of 16°. On the day of the experiment, these trained rats underwent the same acute bout of eccentric training described previously and were killed 2 h after recovery. To assay only the effects of the drug, a PDTC-treated control group was included.

Tissue preparation. All animals were anesthetized with pentobarbital sodium (50 mg/kg); after exsanguinations, the skeletal muscle deep portion of the vastus lateralis (DVL) muscle was immediately excised from both legs, freeze-clamped between aluminum tongs precooled with liquid nitrogen, and stored at −80°C for later analyses.

Determination of nitrate concentration. Nitrate concentration was measured in DVL muscle by using an assay kit (Oxford Biomedical Research, Oxford, MI).

RNA extraction and RT-PCR analysis. Total RNA was isolated from DVL muscle by using the SV total RNA isolation system (Promega, Madison, WI) and quantified by the fluorescent method Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Residual genomic DNA was removed by incubating RNA with DNase I RNase-free DNase (Ambion, Austin, TX). RNA integrity was confirmed by formaldehyde gel electrophoresis. First-standard cDNA was amplified using high-capacity cDNA archive kit (Applied Biosystems, Weiterstadt, Germany). The negative control (no transcriptase control) was performed in parallel. Real-time PCR was carried out under optimal conditions with the following PCR amplification mixture (20 μl total): 2× QuantiTec SYBER green PCR master mix (Applied Biosystems) and 0.3 μM of forward and reverse specific primers. The sets of PCR primers used for the analysis of the mRNA abundance were as follows: 5′-CGATCGGCTTTGTTAGAC-3′ and 5′-AGGCAATGCGCGTTGCAG-3′ for nNOS, 5′-CCAGGTCACA- CGGGTACTC-3′ and 5′-GCTCTTCTGCGGATGTCTTG-3′ for iNOS, 5′-TGGACGACACAGAGTTAAGAAC-3′ and 5′-GGGCGG- CAAGAGGATACCA-3′ for eNOS, and 5′-CCTCATGACGATTGTGAG- GAGAGGAC-3′ and 5′-CGGAGTCAGCGGAACATG-3′ for DVL.

Western blot. Samples of cytosolic fraction containing 50–75 μg of protein were separated by SDS-PAGE (9–14% acrylamide) and transferred to PVDF membranes. Nonspecific binding was blocked by preincubation of the PVDF membranes in PBS containing 5% BSA for 1 h. The membranes were then incubated with a 1:200 dilution of the primary antibody overnight at 4°C with appropriate antibodies. Antibodies against nitrotyrosine and iNOS (130 kDa) were purchased from BIOMOL International (Plymouth Meeting, PA); antibodies against nNOS (155 kDa) and eNOS (133 kDa) were purchased from Abcam (Cambridge, UK); antibodies against IkB-α (41 kDa), phospho-IkB-α (pIkBα) (40 kDa), IKK-α (85 kDa), and phospho-IKK-α (pIKKα) (85 kDa) were purchased from Cell Signaling Technology (Beverly, MA). Bound primary antibody was detected with the use of a peroxidase-conjugated secondary antibody (Dako Cytomation, Glostrup, Denmark) and with the use of an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The density of the specific bands was quantitated with an imaging densitometer. The blots were stripped in 6.25 mM Tris, pH 6.7, 2% SDS, and 100 mM mercaptoethanol at 50°C for 15 min and probed again for anti-β-actin antibodies (Sigma-Aldrich, Madrid, St. Louis, MO) (42 kDa) to verify equal protein loading in each lane.

EMSAs. Nuclear extracts were prepared from skeletal muscle by the method of Dignam et al. (13) with modifications (22). To assess the purity of the nuclear extracts, Western blots for several enzyme-specific subcellular fractions were run. No contamination with the membrane and the cytosolic or mitochondrial fractions occurred. Binding activity of NF-κB was determined in nuclear extracts of DVL by means of EMSA, as previously reported (21). Oligonucleotides were end-labeled with [γ-32P]ATP to a specific activity of >5 × 107 cpm/μg for DNA-NF-κB consensus 5′-AGTGGAGGGACTTCCAG- GCCG-3′ and 3′-TCACACTCCCCCAAGGTCCG-5′. Nuclear extract (40 μg) was incubated for 20 min at room temperature in binding buffer in the presence of ~1 ng of labeled oligonucleotide (~250 μCi (GE Healthcare Biosciences, Uppsala, Sweden)). To verify that the results from EMSA analysis did not arise from nonspecific binding, competition experiments were also carried out with a negative control (cold probe) and muscle sample + non-specific competitor. The nonspecific competitor reaction used an oligonucleotide with a different sequence to the specific oligonucleotide listed above. In this instance, SP1 oligonucleotide (Promega) was used. If the signal was specific, addition of nonradiolabeled specific competitor would decrease the signal intensity. An additional aliquot was prepared and loaded onto the gel, which contained all reagents with the exception of sample (negative control or cold probe). Protein-DNA complexes were separated from the free DNA probe by electrophoresis through 6% native polyacrylamide gels containing 10% ammonium persulfate and 0.5× Tris-borate-EDTA buffer. Gels were dried under vacuum on Whatmann DE-81 paper and exposed for ~48–72 h to Amersham Hyperfilms at ~−80°C.

Chromatin immunoprecipitation assay. Chromatin in DVL muscle was fixed and immunoprecipitated according to Borrás et al. (7). Briefly, ~100 mg of diced DVL muscle were treated with 1% (vol/vol) formaldehyde for 12 min to cross-link the chromatin, and the
reaction was stopped by adding glycine to a final concentration of 0.125 M. After centrifugation at 1,500 g for 5 min, the cell pellet was resuspended in cell lysis buffer (85 mM KCl, 0.5% Nonidet P-40, 5 mM HEPES, pH 8.0) supplemented with protease inhibitor cocktail (Sigma-Aldrich), incubated on ice for 15 min, and centrifuged at 3,500 g for 5 min to pellet the nuclei. The pellet was resuspended in nuclear lysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris - HCl, pH 8.1) at a ratio 1:1 (vol/wt) relative to the initial tissue weight, incubated on ice for 10 min, aliquoted in 1-ml fractions, and stored at −80°C until use for chromatin immunoprecipitation (ChIP) assay.

Cross-linked chromatin (1 ml of each sample) was sonicated on ice by 8–10 bursts of sonication at 29% amplitude in a Vibra-Cell (Sonics & Materials, Newtown, CT). The average size of the chromatin fragments obtained was ~100–500 bp, and fragment sizes were checked by agarose gel electrophoresis. The sonicated chromatin was centrifuged at 14,000 g for 10 min, and the supernatants, which contained soluble chromatin fragments, were diluted 10-fold with dilution buffer (165 mM NaCl, 0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris - HCl, pH 8.0) supplemented with protease inhibitor cocktail. The diluted chromatin fractions were preextracted by adding 30 μl/ml protein A/G agarose (GE Healthcare Biosciences), previously blocked for 1 h with 100 μg/ml λ-DNA, 500 μg/ml tRNA, and 1 mg/ml BSA, and kept for 4 h at 4°C on a rotating plate. The suspensions were then centrifuged at 14,000 g for 30 s to remove nonspecifically bound chromatin fragments. Aliquots from the supernatant (equivalent to 50 μg of DNA) were obtained, incubated with 2 μg of specific antibodies against the NF-κB p65 subunit and an antibody against RNA polymerase II (sc-899) (both from Santa Cruz Biotechnology, Santa Cruz, CA), and left overnight at 4°C under rotation. The samples were then incubated with 50 μl of blocked protein A/G agarose under rotation for an additional 4 h. The immune-complex was recovered by centrifugation at 14,000 g for 30 s and washed as described previously (43). An aliquot of the cross-linked chromatin was treated as above but in the absence of the antibody (no Ab fraction); the first supernatant, after it was precleared with protein A/G agarose, was saved as the input fraction. The immunoselected chromatin was eluted from the protein A/G agarose, was saved as the input fraction. The immunoselected chromatin was eluted from the protein A/G agarose in two consecutive steps by adding 100 μl of elution buffer (1% SDS, 100 mM NaHSO4−) each time, with 30 s of vigorous vortexing. The two supernatants were combined (immunoprecipitated fraction) and incubated at 65°C overnight to reverse formaldehyde cross-links. The DNA from all the samples was purified with a PCR purification kit (Qiagen, Germantown, MD) and used for PCR analysis of the target genes.

**PCR analysis of the immunoprecipitated chromatin.** After DNA purification, the input, immunoprecipitated, and no Ab fractions were analyzed by PCR with appropriate primer pairs to amplify products of 200–300 bp in length, corresponding to either the promoter or the coding regions of the target genes. Primers for PCR analysis of promoter regions were as follows: 5’-TTTGGGAGTCTTCGGTG-GACA-3’ and 5’-ATCCATGGTACCCCGAAAG-3’ for nNOS, 5’-CCTCCCTCCCTAGGTGAGTCC-3’ and 5’-CCCACTAGGGTGCAGAGTTC-3’ for iNOS, 5’-AAAGACGCTGCTTGGGATCC-3’ and 5’-GAGTTGGCCCTGGGAATTTT-3’ for HPRT. Primers used for the analysis of the coding regions were as follows: 5’-GCCGCTCTCTAACCTCTC-3’ for eNOS, and 5’-CAGACGCG-GCCAGTACATTT-3’ and 5’-AAGTTGGGCCTGGGAATTTT-3’ for HPRT. Primers used for the analysis of the coding regions were as follows: 5’-GCCGCTCTCTAACCTCTC-3’ for eNOS, and 5’-CAGACGCG-GCCAGTACATTT-3’ and 5’-AAGTTGGGCCTGGGAATTTT-3’ for HPRT. Primers used for the analysis of the coding regions were as follows: 5’-GCCGCTCTCTAACCTCTC-3’ for eNOS, and 5’-CAGACGCG-GCCAGTACATTT-3’ and 5’-AAGTTGGGCCTGGGAATTTT-3’ for HPRT. PCR fragments were size-fractionated by 2% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide.

**Statistical analysis.** Results are shown as means ± SE. Data for EMSA, Western blots, and RT-PCR are presented as percentages from control values. Data were analyzed using one-way ANOVA. Post hoc comparisons were carried out by the Newman-Keuls test. A value of P < 0.05 was regarded as significant. All calculations were performed by using the SPSS 14.0 statistical software (SPSS, Chicago, IL).

**RESULTS**

The different parameters analyzed in the PDTC-treated control group did not show significant differences compared with control values.

Superoxide and NO are known to bind to form peroxynitrite. Peroxynitrite and/or peroxynitrite-derived intermediates can free nitrate or protein-bound tyrosine residues to form 3-nitrotyrosine. The detection of protein-bound 3-nitrotyrosine is often used as a marker of inflammation and NO production. Figure 1A shows the pattern of nitrated proteins in muscle. Detected immunoreactive bands indicate a significant increase of endogenous tyrosine nitrination after the acute bout of eccentric exercise compared with that shown in control animals (P < 0.05). PDTC treatment and exercise training partly suppressed this increase (P < 0.05). However, trained rats showed a higher decrease in the nitrated protein than shown in the acute and PDTC-treated groups (P < 0.05). Similar effects were observed when the nitrate concentration in muscle was analyzed (Fig. 1B).

Figure 2 depicts the EMSA for NF-κB binding in the various groups of rats. A single bout of eccentric exercise caused the expected significant activation of NF-κB (P < 0.05). Administration of PDTC attenuated the exercise effect (P < 0.05), although these rats still had higher levels of NF-κB binding than control rats. Trained rats showed a significant decrease in the NF-κB binding activity to NF-κB consensus sequence (P < 0.05), lower than that observed in the acute and PDTC-treated groups (P < 0.05).

Because it has been documented that activation of NF-κB correlates with rapid proteolytic degradation of IkBα, the next step was to examine whether increased NF-κB binding was associated with phosphorylation and degradation of IkBα. At rest, most of the IkBα protein is in the nonphosphorylated form and not much is present in the phosphorylated form. DVL muscle from acute exercised rats showed a marked reduction of IkBα (P < 0.05) and a corresponding increase in pIkBα (P < 0.05). These exercise effects were partially prevented in PDTC-treated and trained rats (P < 0.05) (Fig. 3).

Because IkBα phosphorylation is controlled by IKK in most cells, we measured the content of IKKα and its active form (pIKKα) in rat DVL muscle. The acute exercised group showed a marked reduction of IKKα and an increase in pIKKα (P < 0.05) (Fig. 4). Similarly, training caused a significant decrease in IKKα and increase in pIKKα protein content compared with that shown in the acute exercised group (P < 0.05). Figure 4 also shows that both PDTC-treated and trained rats showed a lower decrease (P < 0.05) in IKKα levels and lower increase (P < 0.05) in pIKKα content than the acute group.

To determine whether exercise modulation of gene expression. RT-PCR was performed. The mRNA level of the three NO isoforms was significantly increased after acute eccentric exercise (P < 0.05). Although PDTC-treated and trained animals also showed a significantly increased expression (P < 0.05), this was significantly lower than shown in the acute group (P < 0.05) (Table 1). Protein content of NO
isoforms was studied by Western blot (Fig. 5). A significant increase of the three isoforms was observed in acutely exercised rats compared with that shown in the rested animals ($P < 0.05$). This effect was partially abolished with the administration of PDTC and with training ($P < 0.05$).

To determine the in vivo nuclear protein binding to the nNOS, iNOS, and eNOS promoter regions, ChIP assays were performed with an affinity-purified antibody directed against p65 (Fig. 6). DNA was extracted from the input, immunoprecipitated, and no Ab fractions; equal amounts from each fraction were amplified using primers specific for the nNOS, iNOS, and eNOS promoter regions. Binding was determined by the relative intensity of ethidium bromide fluorescence when compared with the input control fraction. Our data clearly show that binding of NF-κB to the nNOS, iNOS, and eNOS promoters was evident in all experimental groups after an acute bout of eccentric exercise, whereas it was undetectable in control rats. However, binding was significantly lower ($P < 0.05$) in the skeletal muscle of PDTC-treated rats and trained animals (Fig. 6). Binding to the HPRT promoter (used as a negative control) was not observed (Fig. 6), indicating specific binding of NF-κB to all of the NOS isoforms. When the same cross-linked chromatin samples used for the ChIP assay with the anti-p65 antibody were also used for ChIP with an antibody against RNA polymerase II, no signal was observed in control rats. However, after an acute bout of exercise, the RNA polymerase was bound to the coding region of the NOS isoform genes in the different experimental groups, providing an excellent internal control of real-time transcription.

**DISCUSSION**

Eccentric exercise has been reported to damage skeletal muscle in a fiber-specific manner, and it is known that oxidative stress and inflammation play a role in the progression of muscle fiber injury after the initial mechanical insult (16). Thus eccentric contraction is accompanied by enhanced production of ROS (36), which, when combined with enhanced NO production, are likely to augment nitratated protein production.
This was confirmed in our study by the significant increase in nitrate concentration and protein tyrosine nitration in muscle. Increased production of NO resulted from the higher expression of the different NOS isoforms, with augmented mRNA level and protein concentration of nNOS, iNOS, and eNOS.

Activation and infiltration of leukocytes, predominantly neutrophils, monocytes, and macrophages, occur in skeletal muscle after exercise. These nonmuscle cells could be hypothesized to contribute to the changes in NOS expression and NF-κB activation observed in the present study. However, in rats subjected to the same eccentric exercise protocol, it has been reported that both neutrophils and ED1 macrophages were significantly elevated at 24 h postexercise, but no significant change was detected in the first 6 h postexercise (50).

Recent data also confirm that myeloperoxidase content is significantly increased only at 3–5 days after eccentric exercise-induced muscle damage (10). Similar results, with progressive increases in myeloperoxidase, which reach no significance until at least 24 h, have been reported with other exercise protocols (38).

Previous data have shown that, in working muscles by exercise, transcriptional regulation is crucial in the excessive NO production primarily by the iNOS isoform (40). The process of iNOS expression involves nuclear translocation of

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**Fig. 3.** Western blot analyses of IκBα and phospho-IκBα (pIκBα) in the cytosolic fraction of deep vastus lateralis skeletal muscle. Left: representative Western blots; n = 8 for each experimental group. Right: results shown as percentage of control values (means ± SE). *P < 0.05 vs. control. **P < 0.05 vs. acute.

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**Fig. 4.** Western blot analyses of IκB kinase α (IKKα) and phospho-IKKα (pIKKα) in the cytosolic fraction of deep vastus lateralis skeletal muscle. Left: representative Western blots; n = 8 for each experimental group. Right: results shown as percentage of control values (means ± SEM). *P < 0.05 vs. control. **P < 0.05 vs. acute.
NF-κB (10, 14, 27). Moreover, the activation of IKKα/β is tightly coupled to NF-κB activity. Consistent with the possible involvement of NF-κB in muscle damage, previous reports have shown prolonged increases in NF-κB binding activity in DVL muscles after exhaustive exercise (2, 26). In the present study, an acute bout of eccentric exercise elevated NF-κB binding in rat DVL muscle, and this effect was accompanied by a cascade of events in the exercised muscle, including enhanced IKK and IκBα phosphorylation and decreased IκBα content in the cytosol, indicating the importance of the NF-κB signaling pathway in response to acute eccentric exercise.

PDTC has been shown to block NF-κB in a range of cell types and in response to diverse stimuli (26, 41, 44). In our study, PDTC-treated acutely exercised rats showed lower NF-κB binding in the muscle nuclear extract, whereas IκBα phosphorylation and degradation induced by acute exercise were almost abolished. The mechanisms involved in PDTC inhibition of NF-κB are not entirely clear. PDTC suppression of NF-κB binding in nonneuronal tissues is thought to be due to inhibition of the release or degradation of IκB through either its metal chelating or antioxidative properties (49). In any case, PDTC administration also reduced the marked augment in tyrosine protein nitration resulting from acute exercise, supporting the regulatory role of NF-κB in NO production, a fact further confirmed by the reduced expression of the different NOS isoforms.

Most of the proteins encoded by NF-κB target gene participate in stress and inflammatory responses, such as iNOS, which is known to have NF-κB binding sites on its promoter (53). Our data showed that, after an acute bout of eccentric exercise, NF-κB was bound to the iNOS promoter, resulting in transcription of the gene, as demonstrated by binding of RNA polymerase II to the coding region of the iNOS gene. This would contribute to explaining the threefold increase in the expression of the iNOS mRNA and protein. Thus NF-κB seems to be a key molecular mechanism for iNOS mRNA induction in skeletal muscle, and its activation seems to be a beneficial mediator of exercise-induced adaptations to cellular stress and inflammation.

A link between NF-κB and iNOS has been clearly established in different models, including exercise (30), but the role of NF-κB in the regulation of eNOS or nNOS in metabolic disease states or exercise is less clear. In rat intestine, evidence suggests that nNOS acts to impair NF-κB activation, which then suppresses iNOS levels (41). nNOS expression has been also reported to be reduced in skeletal muscle of individuals with Type 2 diabetes (8), whereas IκBα/NF-κB signaling is enhanced (47). Although previous studies demonstrated the presence of NF-κB cis-regulatory elements only in the promoter region of the iNOS gene (35), we have also found sequences of this type in the promoter of the nNOS and eNOS genes using RNA pol-ChIP experiments, providing first-time evidence that NF-κB interacts in vivo with the promoter of nNOS and eNOS genes in response to acute exercise. More-

### Table 1. mRNA levels of nNOS, iNOS, and eNOS measured by real-time PCR in deep vastus lateralis skeletal muscle

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<tr>
<td>nNOS, %</td>
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<td>98 ± 13</td>
<td>242 ± 15*</td>
<td>188 ± 18†</td>
<td>196 ± 15†</td>
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<td>iNOS, %</td>
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<td>305 ± 16*</td>
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<td>218 ± 14†</td>
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<tr>
<td>eNOS, %</td>
<td>100 ± 11</td>
<td>99 ± 13</td>
<td>208 ± 19*</td>
<td>155 ± 8†</td>
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Values are means ± SE. Levels of nNOS, iNOS, and eNOS mRNA were normalized against hypoxanthine phosphoribosyl-transferase-1 (HPRT) rRNA and presented as fold change of duplicate samples from 2 separate experiments for each experimental group (n = 8). C, control; CP, control with pyrrolidine dithiocarbamate (PDTC); A, acute; AP, acute with pyrrolidine dithiocarbamate (PDTC); AT, acute with training. *P < 0.05 vs. C. †P < 0.05 vs. A.

Fig. 5. Western blot analyses of nNOS, iNOS, and eNOS in the cytosolic fraction of deep vastus lateralis skeletal muscle. Left: representative Western blots; n = 8 for each experimental group. Right: results shown as percentage of control values (means ± SE). *P < 0.05 vs. control. †P < 0.05 vs. acute.
over, both nNOS and eNOS mRNA levels and mature protein abundance increased with an acute bout of eccentric exercise, indicating that these isoforms are, in fact, not only constitutively expressed in skeletal muscle but their activity is inducible when the metabolic demand of the muscle increases. In support of this result, a 45-min exhaustive exercise bout increased NOS activity in rat skeletal muscle, with a contribution by both nNOS and eNOS (42). The immunohistochemical association of nNOS with the sarcolema suggests that this isoform influences blood delivery (29, 31, 32), glucose uptake, and excitation-contraction coupling, whereas eNOS expression is associated with the mitochondria in rodents and correlates with mitochondrial respiration and therefore most likely influences oxidative phosphorylation (28). On the other hand, an increase in eNOS expression during exercise is also considered an important vascular adaptation to laminar shear stress, the tangential force exerted by flow over the surface of the endothelium (18).

PDTC administration not only partially inhibited iNOS but also nNOS and eNOS promoter activity and subsequent iNOS, nNOS, and eNOS gene transcription in response to an acute exercise bout. Our results reconfirm that the expression of the three NOS isoforms studied is tightly regulated via NF-κB during exercise, with strong reduction after treatment with the antioxidant/anti-inflammatory agent PDTC.

We also tested the potential effects of 8 wk of eccentric training on the changes in gene expression of NOS isoforms and NF-κB binding induced by acute exercise. Whereas a large number of studies have analyzed the effects of chronic training in preventing oxidation and/or inflammation in exercise, there is little information on the effect of a training program prior to acute bouts of exercise (14, 27) and particularly on NOS isoform expression in skeletal muscle in this situation. Regarding the influence of exercise training on muscle NO production, the reduction in nitrotyrosine formation indicated that 8 wk of exercise prevented the production of reactive nitrogen species generated from the activity of NOS after acute physical exercise. Because NO has multiple roles in the circulatory and metabolic response to an acute bout of exercise, animal studies have flagged adaptations in the skeletal muscle NO system as being responsible for some of the metabolic benefits of training (28). Therefore, it is not surprising that this system adapts in response to training and that such adaptations may contribute to enhanced exercise capacity and reduced inflammation. In fact, relatively small changes in generation of NO appear to lead to sensitive changes in several other substances that potentially influence cellular responses (24).

Evidence has also shown that a training program can exert an inhibitory effect on NF-κB-DNA binding (9, 14, 27). The effects observed after a bout of acute exercise on the NF-κB signaling pathway were ameliorated by 8 wk of submaximal eccentric training, which resulted in an attenuated activation of NF-κB and lower changes in IkBα, pIkBα, IKKα, and pIKKα content. In accordance with these data, the concomitant measurement of NOS isoform mRNA levels; nNOS, iNOS, and eNOS protein content; and NF-κB interaction with target NOS isoform promoters clearly showed that the levels of nNOS, iNOS, and eNOS expression in skeletal muscle from trained rats decreased significantly compared with the acute group. Therefore, our study reinforced and extended the work of others (17, 25, 28, 51) who have demonstrated that adaptations (e.g., vascular, antioxidant, or anti-inflammatory) are associated with
regular physical training, leading to the suggestion that transcriptional regulation of iNOS, nNOS, and eNOS isoforms by NF-κB plays a major role in training-induced adaptations.

In summary, our results provided a direct link between the NF-κB signaling cascade and NOS isoform gene expression in skeletal muscle following acute eccentric exercise. To our knowledge, we are the first to report the presence of NF-κB bound to nNOS, iNOS, and eNOS promoters after an acute bout of exercise, suggesting a modulation of the expression of the three NOS isoforms by this transcription factor. Nevertheless, this does not exclude the contribution of other transcription factors activated by additional pathways. Furthermore, because NF-κB may be a key transcription factor in the training-induced adaptation process, this regulatory mechanism seems to govern not only the responses to acute eccentric bout but also other important biological processes in skeletal muscle such as adaptation to exercise.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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