Regulation of nitric oxide production in limb and ventilatory muscles during chronic exercise training

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Vassilakopoulos, T., G. Deckman, M. Kebbewar, G. Rallis, R. Harfouche, and S. N. A. Hussain. Regulation of nitric oxide production in limb and ventilatory muscles during chronic exercise training. Am J Physiol Lung Cell Mol Physiol 284: L452–L457, 2003.—In this study, we evaluated the differential influence of chronic treadmill training (30 m/min, 15% incline, 1 h/day, 5 days/wk) on nitric oxide (NO) production and NO synthase (NOS) isoform expression as well as 3-nitrotyrosine formation (footprint of peroxynitrite) both in limb (gastrocnemius) and ventilatory (diaphragm) muscles. A group of exercise-trained rats and a control group (no training) were examined after a 4-wk experimental period. Exercise training elicited an approximate fourfold rise in gastrocnemius NOS activity and augmented protein expression of the endothelial (eNOS) and neuronal (nNOS) isoforms of NOS to ∼480% and 240%, respectively. Qualitatively similar but quantitatively smaller elevations in NOS activity and eNOS and nNOS expression were observed in the diaphragm. No detectable inducible NOS (iNOS) protein expression was found in any of the muscle samples. Training increased the intensity of 3-nitrotyrosine only in the gastrocnemius muscle. We conclude that whole body exercise training enhances both limb and ventilatory muscle NO production and that constitutive and not iNOS isoforms are responsible for increased protein tyrosine nitration in trained limb muscles.

skeletal muscle; peroxynitrite; oxygen radicals

Nitric oxide (NO), an endogenously produced molecule with numerous biological functions, is synthesized inside skeletal muscle fibers by the neuronal (nNOS) and the endothelial (eNOS) nitric oxide synthases (12, 13). The nNOS isoform directly associates with the dystrophin complex and is localized in close proximity to the sarcoplemma of mainly type II fibers (8), whereas the eNOS isoform has a mitochondrial localization and is, therefore, abundantly expressed in type I fibers (13). Although very low levels of the inducible NOS (iNOS) isoform may be expressed in skeletal muscles of normal mammals, the expression of this isoform in limb and ventilatory muscles rises significantly and in a tran-

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supplied through active transport from the extracellular space, protein degradation, and recycling of L-citrulline to L-arginine. This recycling occurs via a two-step enzymatic process that requires the activities of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) enzymes (26). Thus it is possible that chronic exercise training may elicit an increase in muscle NO production through the induction of iNOS and upregulation of AS and AL expression inside muscle fibers.

Exercise is also accompanied by enhanced production of reactive oxygen species inside muscle fibers, which, when combined with enhanced NO production, leads to the formation of peroxynitrite, which in turn targets proteins and lipids and leads to posttranslational modifications and inactivation of various enzymes, including those involved in energy production, fatty acid metabolism, and the defense against oxidative stress (6). One of the posttranslational protein modifications elicited by peroxynitrite is nitration of tyrosine residues and loss of protein function as a result of 3-nitrotyrosine formation (2). Whether chronic exercise training alters protein tyrosine nitration of leg and ventilatory muscles has never been addressed.

The main aim of this study was to assess the influence of whole body chronic exercise training on NO production and NOS isoform production in both the limb and ventilatory muscles. We have also assessed whether chronic exercise training influences the expression of enzymes involved in recycling of L-arginine and whether protein tyrosine nitration (footprints of peroxynitrite formation) inside limb and ventilatory muscles is influenced by whole body chronic exercise training.

MATERIALS AND METHODS

Materials. Reagents for protein measurement were purchased from Bio-Rad (Hercules, CA). Gels and loading buffers for immunoblotting were obtained from Novex (San Diego, CA). Chemicals were purchased from Sigma Chemical (St. Louis, MO). Primary monoclonal anti-eNOS, anti-nNOS, and anti-iNOS antibodies were obtained from Transduction Laboratories (Lexington, KY). Monoclonal anti-3-nitrotyrosine was obtained from Cayman Chemical (Ann Arbor, MI). Secondary antibodies for immunoblotting were obtained from Transduction Laboratories. Reagents for enhanced chemiluminescence detection were obtained from Chemicon (Temecula, CA).

Animal preparation and experimental protocol. The Animal Research Committee of McGill University approved the procedures used in this study. Pathogen-free, male Sprague-Dawley rats (250–300 g) were studied 1 wk after arrival. The animals were then randomly assigned to one of two groups. Group 1 rats served as control (sedentary) and were allowed cage activity only. Group 2 (exercise) rats were exposed to 4 wk of treadmill exercise training after a 2-wk period of conditioning (treadmill running 10 min/day at a speed of 30 m/min and 0% incline). After the conditioning period, the incline of the runway was increased by 5% every 4 days, and the duration of running was increased by 5 min/day, so that the animals were finally exercising for 1 h/day at 30 m/min and 15% incline (training period). The training period was maintained for 4 wk. All animals were monitored during the running session. One day after the end of the exercise training period, the animals were euthanized with an overdose of pentobarbital sodium. The chest was then opened, and the diaphragm was quickly excised and frozen in liquid nitrogen. In addition, the gastrocnemius muscle was also excised and frozen.

L-citrulline assay. Frozen muscle tissues from each animal were homogenized in a small volume (wet weight) of homogenization buffer (10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithiothreitol, 0.32 mM sucrose, 1 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml apronitin, and 10 μg/ml pepstatin A, pH 7.4). The crude homogenates were centrifuged at 4°C for 15 min at 10,000 rpm. The supernatant (50 μl) was added to 10-ml prewarmed (37°C) tubes, each containing 100 μl of reaction buffer of the following composition (in mM): 50 KH2PO4, 60 valine, 1.5 NADPH, 10 flavine adenine dinucleotide, 1.2 MgCl2, and 2 CaCl2, as well as 1 mg/ml bovine serum albumin, 1 μg/ml calmodulin, 10 μM tetrahydrobiopterin, and 25 μl of 120 μM stock l-arginine-2,3-3H (150–200 counts·min−1·pmol−1). The samples were incubated for 50 min at 37°C, and the reaction was terminated by the addition of 400 μl of Dowex 50w resin (0.5% cross-linked, Na+ form) to obtain free L-[3H]citrulline for the determinations of enzyme activity, 2 ml of Dowex 50w resin (10% cross-linked, Na+ form) were added to eliminate excess l-arginine-2,3-3H. The supernatant was removed and examined for the presence of L-[3H]citrulline by liquid scintillation counting. Enzyme activity was expressed in picomoles of L-citrulline produced per milligram of protein per minute. Protein was measured by the Bradford technique with bovine serum albumin as standard. NOS activity was also measured in the presence of 1 mM of Nω-nitro-l-arginine methyl ester (NOS inhibitor). Total specific muscle NOS activity was calculated as the difference between that measured in the presence and absence of Nω-nitro-l-arginine methyl ester.

Immunoblotting. Crude gastrocnemius and diaphragmatic muscle homogenates (80 μg of total protein/sample) were mixed with sample buffer, boiled for 5 min at 95°C, and then loaded onto 8 or 12% gradient Tris-glycine SDS polyacrylamide gels and separated by electrophoresis (150 V, 30 mA for 2.5 h). Proteins were transferred electrophoretically (25 V, 375 mA for 2.5 h) to nitrocellulose membranes, blocked with 5% nonfat dry milk (1 h), and subsequently incubated overnight at 4°C with primary antibodies. The eNOS, nNOS, and iNOS proteins were detected with selective monoclonal antibodies (1:500 for each antibody) with lysates of endothelial cells, pituitary cells, and cytokine-activated macrophages as positive controls, respectively. AS and AL proteins (1:1,000) were detected with polyclonal antibodies that were raised in rabbits against recombinant proteins and were used previously to detect these proteins in the diaphragm and other rat tissues (19). The formation of 3-nitrotyrosine was detected with a monoclonal antibody as previously indicated (5). After three 10-min washes with wash buffer on a rotating shaker, the PVDF membranes were further incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. Specific proteins were detected with a chemiluminescence kit (Chemicon). The blots were scanned with an imaging densitometer, and optical densities (OD) of positive protein bands were quantitated with ImageQuant software (Molecular Dynamics). Predetermined molecular weight standards (Novex) were used as markers. For nitrated proteins, total 3-nitrotyrosine OD was calculated for each sample by adding the OD of the individual positive protein bands. Specificity of anti-3 nitrotyrosine antibodies was evaluated by preincuba-
tion of each primary antibody with either 10 mM of nitrotyrosine or 10-fold excess of peroxynitrite-tyrosine nitrated bovine serum albumin (generously provided by Dr. H. Ischiropoulos, University of Pennsylvania).

**Statistical analysis.** All values are expressed as means ± SE. Comparisons between groups were carried out using Wilcoxon’s matched pair test. A P value of <0.05 was regarded as a statistically significant difference.

**RESULTS**

Figure 1 illustrates the influence of exercise training on gastrocnemius and diaphragm NOS activities. Muscle NOS activity measured at the end of the 4-wk training period showed an ~400% increase in the gastrocnemius and an ~50% increase in the diaphragm (Fig. 1). P < 0.05 compared with sedentary rats.

Immunoblotting of muscle lysates with selective anti-nNOS and anti-eNOS antibodies detected positive protein bands with apparent molecular masses of 165 and 140 kDa, respectively, in the gastrocnemius muscle samples of sedentary rats (Fig. 2A). The intensities of gastrocnemius nNOS and eNOS protein bands rose significantly after chronic exercise training to ~240% and 480% of those measured in sedentary rats, respectively (P < 0.05, Fig. 2). Similarly, exercise training elicited a significant rise in diaphragmatic nNOS and eNOS expression but to a smaller extent than that found in the gastrocnemius (Fig. 3).

No detectable iNOS protein expression was found in the gastrocnemius and diaphragmatic muscle samples obtained from sedentary and trained rats (Fig. 4A). Selective anti-AS and anti-AL antibodies were detected in gastrocnemius and diaphragmatic sample positive protein bands with apparent molecular masses of 48 and 47 kDa, respectively (Fig. 4B). Exercise training had no effect on the intensity of AS and AL protein in the gastrocnemius (Fig. 4B) and the diaphragm (results not shown).

Anti-3-nitrotyrosine antibody identified several positive bands in the gastrocnemius and diaphragms of sedentary rats. In the gastrocnemius, tyrosine-nitrated protein bands of 190, 78, 60, 46, 40, and 35 kDa were detected (Fig. 5A). The intensities of the 190-, 78-, 60-, and 40-kDa tyrosine-nitrated protein bands rose significantly in the gastrocnemius muscles of trained rats (P < 0.05 compared with sedentary rats). By comparison, anti-3-nitrotyrosine antibody detected five positive tyrosine-nitrated protein bands of 190, 78, 60, 46, and 35 kDa in diaphragm samples of sedentary rats (Fig. 5). Chronic exercise training had no effect on the intensity of these proteins (Fig. 5).

**DISCUSSION**

The main findings of our study are as follows: 1) Chronic whole body exercise training elicited significant upregulation of gastrocnemius muscle NOS activity and nNOS and eNOS protein expressions. A qualitatively similar but quantitatively smaller rise in muscle NOS activity and nNOS and eNOS protein expression was observed in the diaphragm. 2) Chronic exercise training was not associated with iNOS induction in limb and ventilatory muscles and did not elicit
significant changes in AS and AL expression in these muscles.

3) There was abundant protein tyrosine nitration in both the diaphragm and the gastrocnemius of sedentary rats; however, chronic exercise training was associated with enhanced protein tyrosine nitration only in the gastrocnemius and not in the diaphragm.

Regulation of NOS expression and exercise training.
To our knowledge, we are the first to report concurrent measurements of NOS activity and protein expression in limb and ventilatory muscles in response to chronic whole body exercise training. Chronic exercise training resulted in increases in both gastrocnemius and diaphragmatic eNOS and nNOS protein expression with a commensurate rise in NOS activity.

Only recently was the effect of chronic exercise training on limb muscle NO production evaluated. Treadmill exercise in rats (8 wk) has been shown to cause a fourfold increase in nNOS expression and a twofold increase in eNOS expression in the soleus muscle (4). The functional relevance of these changes in NOS expression to muscle NOS activity in that study is hard to assess because NOS activity was not measured and because NOS activity doesn't always correlate with NOS protein expression (9). In another study, Tatchum-Talom et al. (27) trained rats by having them swim for 3–4 wk and reported a doubling of quadriceps muscle NOS activity and an ~70% increase in nNOS protein abundance in this muscle. These authors, however, did not detect eNOS in either trained or sedentary muscles. Reiser et al. (21) described that 3 wk of artificial limb muscle stimulation produced a significant rise in muscle NOS activity and a concomitant augmentation of muscle nNOS activity.

Our results show that whole body chronic exercise training elicits significant upregulation in both nNOS and eNOS expression of limb and ventilatory muscles. The finding of a proportionally greater increase in eNOS expression in response to exercise training than nNOS expression is qualitatively different from the findings of Balon and Nadler (4) and Tatchum-Talom et al. (27). We speculate that this divergence is due to differences in the types of muscle examined (gastrocnemius, soleus, tibialis anterior, and quadriceps femoris). In rodent muscles, the nNOS and the eNOS isoforms are abundantly expressed inside type II and I fibers, respectively (12, 13). Accordingly, sedentary limb muscles differ significantly in their abundance of nNOS and eNOS proteins, and, therefore, the influence of chronic exercise on NOS expression in these muscles is likely to vary. The type of exercise protocol is another...
induction in limb and ventilatory muscles nor does it evoke upregulation of muscle AS and AL protein expression. Possible explanations for this finding include that, in our study, exercise type and intensity were insufficient to provoke muscle fiber injury, and, consequently, very little secondary leukocyte infiltration, a potential source for iNOS induction, might have occurred. It is also possible that a certain threshold intensity of muscular activity is required before exercise leads to enhanced production of proinflammatory cytokines inside muscle fibers (23). This threshold activity might not have been reached in the exercise program adopted in this study.

Protein tyrosine nitration. Our study reveals that prominent protein tyrosine nitration develops in skeletal muscles of sedentary rats, which is in accordance with recent studies (5). We report here for the first time that the intensity of protein tyrosine nitration in limb muscles is enhanced in response to chronic exercise training. This finding supports (yet cannot prove) the notion that muscle protein tyrosine nitration might be dependent on NO production, since both NOS activity and protein expression were elevated in limb muscles. An interesting observation in our study is that the intensity of specific tyrosine-nitrated proteins increased significantly with exercise training, whereas other tyrosine-nitrated proteins were less sensitive to the effect of exercise training, which suggests that the process of protein tyrosine nitration is highly selective to specific proteins (1, 2, 17, 18). However, the influence of tyrosine nitration on protein function remains, in most cases, unclear. Tyrosine nitration may result in either loss or gain of function or may have no effect (2). Thus whether the increase in protein tyrosine nitration in muscles secondary to chronic exercise training is adaptive or maladaptive or is simply the footprint of oxidative and nitrosative stress with no specific physiological relevance is not known.

The exact contribution of each of the NOS isoforms expressed in skeletal muscle fibers to protein tyrosine nitration in normal and exercised muscle remains speculative. Our experiments suggest that iNOS is not a primary effector in this setting, because no increase in iNOS protein expression was observed secondary to chronic exercise. However, iNOS is the isoform most responsible for protein nitration in normal and septic muscles (5), and the nNOS isoform is involved in protein tyrosine nitration in neurons (3). Thus the role of the various NOS isoforms in protein nitration likely varies with the tissue and condition examined (resting vs. exercise vs. sepsis).

Implications. Enhanced muscle NO production in exercise-trained rats may influence many processes inside skeletal muscle fibers, including glucose metabolism, redox status, contractile performance, and blood flow. Both basal and contraction-stimulated glucose uptakes are enhanced by endogenous NO synthesis (4, 22). Thus one can speculate that elevated nNOS and eNOS expression in response to exercise training represents an adaptive response by muscle fibers to cope with increased demands for glucose uptake. Moreover,
endogenous NO production inside muscle fibers serves an important function as an antioxidant by directly scavenging oxygen radicals and by modifying the influence of oxygen radicals on excitation-contraction coupling. Indeed, NO modulates the activity of sarcoplasmic reticulum ryanodine receptors and Ca$^{2+}$ release through S-nitrosylation of critical cystein residues (25). NO also protects these receptors from the deleterious effects of oxygen radicals that elicit disulfide bond formation of critical cystein residues (24). These effects of NO on sarcoplasmic reticulum Ca$^{2+}$ are likely to improve muscle contractile performance. Finally, enhanced muscle NO production in trained muscles is expected to improve muscle perfusion by inducing direct relaxation of vascular smooth muscles and by inhibiting adrenergic vasoconstriction of muscle resistance vessels (28).

In summary, we have shown that limb and ventilatory muscle NOS expression and activity undergo significant upregulation in response to chronic exercise training. Enhanced muscle NO production was associated with augmented tyrosine nitration of specific proteins with no apparent induction of the iNOS isofrom.

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