Endurance training does not alter the level of neuronal nitric oxide synthase in human skeletal muscle

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Frandsen, U., L. Höffner, A. Betak, B. Saltin, J. Bangsbo, and Y. Hellsten. Endurance training does not alter the level of neuronal nitric oxide synthase (nNOS) content and distribution in muscle was investigated. Seven male subjects performed 6 wk of one-legged knee-extensor endurance training (protocol A). Muscle biopsies, obtained from vastus lateralis muscle in the untrained and the trained leg, were analyzed for nNOS protein and activity as well as immunohistochemical distribution of nNOS and endothelial nitric oxide synthase (eNOS). Muscle biopsies were also obtained from another seven male subjects before and after 6 wk of training by endurance running (protocol B) and analyzed for nNOS protein. No difference was found in the amount of nNOS protein in the untrained and the trained muscle either with protocol A or protocol B (P > 0.05). In protocol A, the activity of nNOS was 4.76 ± 0.56 pmol·mg protein\(^{-1}\)·min\(^{-1}\) in the control leg, and the level was not different in the trained leg (P > 0.05). nNOS was present in the sarcolemma and cytosol of type I and type II muscle fibers, and the qualitative distribution was similar in untrained and trained muscle. The number of eNOS immunoreactive structures and the number of capillaries per muscle fiber were higher (P < 0.05) after training than before. The present findings demonstrate that, in contrast to findings on animals, nNOS levels remain unaltered with endurance training in humans. Evidence is also provided that endurance training may increase the amount of eNOS, in parallel with an increase in capillaries in human muscle.

NITRIC OXIDE SYNTHASE (NOS), an enzyme that synthesizes nitric oxide (NO) from L-arginine, exists in several isoforms. In human skeletal muscle, endothelial NOS (eNOS) is located in the vascular endothelium and neuronal NOS (nNOS) is located in the sarcolemma and cytosol of the muscle fibers, in apparent association with mitochondria (6). NOS has been shown to be of importance in the regulation of several events in skeletal muscle tissue. There is evidence supporting a role for NO in the regulation of muscle blood flow, at least at rest and in recovery from exercise (4, 15). An interaction between NO and superoxide radicals in the regulation of muscle contractile force has been demonstrated (12), and a role for NO has also been implicated in the regulation of mitochondrial respiration (17) as well as in insulin-mediated glucose uptake (14, 21). The importance of NOS for skeletal muscle function is clear, and enhanced contractile activity could be expected to result in an elevated level of the enzyme. In congruence with this hypothesis, Reiser et al. (13) showed that chronic in vivo stimulation of rabbit muscle for 3 wk induced a marked increase in NOS expression and activity in parallel with a fast-to-slow fiber-type transformation. Similarly, NOS expression was found to increase in rat skeletal muscle, after 8 wk of treadmill running (1) and in muscle cell cultures after 2 days of contractile activity induced by electrical stimulation (20). It is not known, however, whether endurance training in humans may alter the level of nNOS in skeletal muscle or whether such an alteration is restricted to oxidative fibers.

In models of isolated skeletal muscle, it has been shown that NO is formed at a higher rate during contraction compared with at rest (1, 20). The amount of NO formed is frequently assessed by measurements of the hydration products of NO in aqueous solutions, nitrite and nitrate. In previous studies on NO formation in skeletal muscle, nitrite and nitrate have been measured in medium surrounding the isolated muscle preparations (2, 20); however, nitrite and nitrate levels have not previously been measured directly in the muscle tissue. Such measurements performed on muscle biopsies before and after an exercise bout, for a trained and an untrained muscle group, could provide an indication of the in vivo formation of NO in humans and whether training affects NO production.

The primary hypothesis of the present study was that endurance training of sedentary human subjects would result in a higher level of skeletal muscle nNOS protein and activity and that this increase mainly would occur in the cytosol of type I fibers. Muscle biopsies obtained before and after training were analyzed for nNOS protein, NOS activity, distribution of nNOS and eNOS, and nitrite and nitrate levels before...
and after exercise. The training protocol, utilizing one-legged knee-extensor exercise, was chosen to allow for training with a well-defined muscle group and to have a matching untrained muscle for control tissue. In addition, for comparative purposes, the effect of training with a larger muscle mass, and thus a greater hormonal response, on muscle nNOS levels was also examined in another group of subjects performing running training for a similar period.

**MATERIALS AND METHODS**

**Protocol A.** Seven healthy male subjects with a mean age of $24 \pm (SE) 1$ yr (range 20–32 yr), mean body weight of 76.1 \pm 2.7 kg (62.0–85.0 kg) and mean maximum oxygen uptake ($V_{O_2 max}$) of $45.4 \pm 1.9$ ml·kg$^{-1}$·min$^{-1}$ (39.2–54.8 ml·kg$^{-1}$·min$^{-1}$) participated in the study. The subjects were informed of any risks and discomfort associated with the experimental procedures before giving their informed consent to participate. The study was approved by the Local Ethical Committee of Copenhagen and Frederiksberg. The workload had been set in previous tests to result in exhaustion after $30 \pm 1$ h. The mean work rate for the subjects could be maintained through all three bouts.

The subjects also performed two 20-min exercise bouts at $60 \pm 1$ h and the control leg 24 h after the last training session. The biopsies were divided into two portions, one of which was frozen directly in liquid nitrogen and the other mounted and frozen in isopentane cooled in liquid nitrogen, which was frozen directly in liquid nitrogen and the other and distilled water to a final volume of 130 µl. The reaction was carried out at $37^\circ$C for 20 min, and the reaction was terminated by the addition of 400 µl of an ice-cold stop buffer (50 mM HEPES and 5 mM EDTA, pH 5.5). Then, 200 µl of resin buffer consisting of 1 g AG 50W-8x (Na$^+$ form) resin in 1 ml of 50 mM HEPES buffer (pH 5.5) were added to the solution, and the resin-containing incubates were transferred to centrifuge tube filters (SPIN-X, Costar) and centrifuged for 1 min at 15,000 g to eliminate excess L-$^{14}$Carginine. Scintillation liquid was added to the eluates, and the eluates were counted in a Packard 2300TR liquid scintillation counter. The enzyme activity is expressed as picomoles of L-citrulline produced per milligram of protein per minute.

Muscle biopsy samples were obtained before the training and 24 h after the 6 wk of training. Several days before and 2 days after the final training session, the subjects performed an intermittent shuttle run test to assess endurance performance. Performance was determined as time to exhaustion.

**Western blotting.** Western blot analysis was performed on freeze-dried, dissected muscle biopsy samples. The proteins were separated according to their molecular mass by SDS-PAGE. Each sample (15 µg) was run in duplicate on two identical gels. Immunoblotting was conducted by using polyvinylidene difluoride membranes (Millipore, Bedford, MA), and the transfer was carried out for 1 h at 225 mA/membrane and 100 V.

The membranes were blocked with 5% nonfat dried milk in 20 mM Tris, 500 mM NaCl, and 1% Tween-20 (pH 7.5) for 1 h, and the membranes were subsequently incubated overnight at $4^\circ$C with the primary nNOS antibody (N31030, Transduction Laboratories, Lexington, KY). After washing, antibody binding was visualized by incubation with a secondary antibody coupled to alkaline phosphatase, followed by detection with enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK). The blots were quantified with densitometric scanning by using a densitometer (Molecular Dynamics Storm 840) with digital analysis software (ImageQuanNT). The densities of the protein bands were quantitated as local average over background, standardized to the local average observed for a rat brain standard so that the different gels could be compared.

**NOS activity.** NOS activity was determined in freeze-dried, dissected biopsies from protocol A with slight modification of the citrulline assay (3). Briefly, the reaction was started by incubation of 10 µl samples (≈200 µg) in a buffer consisting of 32.25 mM Tris·HCl (pH 7.4). 3.75 µM BH$_4$, 0.63 µM flavin adenine mononucleotide, 1.15 mM NADPH, 3.5 µM L-$^{14}$Carginine, 0.7 mM CaCl$_2$, 0.1 µM calmodulin, and distilled water to a final volume of 130 µl. The reaction was carried out at $37^\circ$C for 20 min, and the reaction was terminated by the addition of 400 µl of an ice-cold stop buffer (50 mM HEPES and 5 mM EDTA, pH 5.5). Then, 200 µl of resin buffer consisting of 1 g AG 50W-8x (Na$^+$ form) resin in 1 ml of 50 mM HEPES buffer (pH 5.5) were added to the solution, and the resin-containing incubates were transferred to centrifuge tube filters (SPIN-X, Costar) and centrifuged for 1 min at 15,000 g to eliminate excess L-$^{14}$Carginine. Scintillation liquid was added to the eluates, and the eluates were counted in a Packard 2300TR liquid scintillation counter.

The enzyme activity is expressed as picomoles of L-citrulline produced per milligram of protein per minute.

**Immunohistochemistry.** The immunohistochemical analysis was performed on cross sections (8 µm) cut from frozen muscle samples obtained from the experimental leg before and after training. For the staining of nNOS, the sections were air dried for 5 min, blocked in 1% BSA, and incubated with the primary antibody (B220–1, Eurodagnostica, Malmö, Sweden) at room temperature for 90 min. Binding was visualized by incubation with a secondary antibody coupled to biotin, followed by an streptavidin-FITC complex.

For the staining of eNOS, the sections were fixed with 4% formaldehyde for 5 min. Sections were blocked in 1% BSA and incubated with primary antibody (N30020, Transduction Laboratories) for 90 min at room temperature. For the staining of capillaries, the sections were air dried and stained for...
45 min with primary antibody CD31 (M0823, DAKO A7S, Glostrup, Denmark). Binding was visualized as for nNOS. Type I fibers were identified with a monoclonal antibody to myosin heavy chain I (M-8421, Sigma Chemical, St. Louis, MO).

Muscle nitrite and nitrate. Freeze-dried, dissected muscle biopsy samples were homogenized in doubly distilled water at a concentration of 1 mg/300 µl and centrifuged at 10,000 g for 20 min. The supernatant was saved and centrifuged at 100,000 g for 15 min. The supernatant was then filtered through filters with a molecular mass cutoff of 30 kDa (Millipore, Bedford, MA). Nitrite and nitrate concentrations were measured with chemiluminescence reaction with ozone. Injection volumes of 25 µl for nitrate and 10 µl for nitrite plus nitrate were used. (NOA 280, Sievers, Boulder, CO; Ref. 7).

Citrate synthase activity. The activity of citrate synthase was measured in freeze-dried, dissected muscle biopsy samples obtained from the control and the experimental legs after 6 wk of training. The assay was conducted at 25°C and was performed according to the method described by Lowry and Passonneau (11).

Protein determination. Protein was determined in triplicate by using a colorimetric assay with bichinchoninic acid (18). Albumin was used as standard.

Statistics. Data are presented as means ± SE. In comparing group means for the experimental leg before and after training, a paired Student’s t-test was used, whereas group means for the untrained and trained legs were compared with an unpaired Student’s t-test. Group means for nitrite and nitrate in the untrained and the trained leg before and after exercise were compared by using a two-way analysis of variance. Differences were considered statistically significant when $P < 0.05$.

Fig. 1. Relative neuronal nitric oxide synthase (NOS) protein content determined by Western blotting (A) and NOS activity determined by the citrulline assay (B) in human muscle biopsy samples obtained from vastus lateralis muscle in the experimental leg before (Pre tr) and in the experimental (Post tr) and the control leg (Post utr) after 6 wk of 1-legged knee-extensor endurance training. Values are means ± SE. Neuronal NOS protein content values are expressed relative to a rat brain standard.

Fig. 2. Distribution of neuronal NOS in cross sections of untrained (a) and endurance-trained (b) human skeletal muscle. Note the stronger staining of cytosol and sarcolemma in type I muscle fibers (indicated by *). Original magnification ×400.
RESULTS

Protocol A. Performance, as assessed by the final work rate that the subjects exercised at in a graded one-legged knee-extensor test to exhaustion, was improved from 54.9 ± 4.2 to 66.3 ± 2.7 W (P < 0.05).

After the 6 wk of training, the nNOS protein level in the experimental leg was not different from pretraining values or from values of the control leg (P > 0.05; Fig. 1A).

The calculated V_max for NOS in skeletal muscle homogenates was 102 pmol·mg protein^{-1}·min^{-1}, and the K_m for L-arginine was 1.4 μM. The NOS activity level before training (4.76 ± 0.56 pmol·mg protein^{-1}·min^{-1}) was similar to that of the experimental leg and that of the control leg after training (P > 0.05; Fig. 1B).

Immunohistochemical analysis of muscle samples obtained from the untrained muscle revealed positive nNOS staining in the sarcolemma and in the cytosol of the muscle fibers (Fig. 2). The staining of both the sarcolemmal region and of the cytosol was stronger in the type I fibers compared with type II fibers (Table 1). There was no qualitative difference in the distribution of nNOS staining in muscle samples obtained from the experimental leg before and after training.

Positive eNOS staining was observed in the endothelium of microvessels. There was a higher number of eNOS-immunoreactive structures in the experimental leg with training (an increase from 2.03 ± 0.15 to 2.28 ± 0.4 structures/muscle fiber; P < 0.05). There was also an increase in the number of CD31-immunoreactive microvessels in the experimental leg with training, from 2.67 ± 0.5 to 2.97 ± 0.64 vessels/muscle fiber (P < 0.05).

The concentrations of nitrite and nitrate were similar in the control and the trained leg at rest (P > 0.05; Fig. 3). After 20 min of submaximal, one-legged knee-extensor exercise, the levels of nitrite and nitrate were unchanged (P > 0.05) in both the control and the trained legs.

The activity of citrate synthase in muscle was significantly higher (P < 0.05) in the trained leg (38.4 ± 1.99 μmol·min^{-1}·g dry wt^{-1}) than in the control leg (34.3 ± 1.6 μmol·min^{-1}·g dry wt^{-1}) after the 6-wk training period.

Protocol B. Performance, as assessed by time to exhaustion in an intermittent shuttle-run test, was improved from 10.3 ± 1.2 to 12.9 ± 1.4 min (P < 0.01).

The relative amount of nNOS protein in the muscle was similar before and after the 6 wk of training (P > 0.05; Fig. 4).

DISCUSSION

The present study examined the effect of endurance training on the protein level, activity, and distribution of nNOS in human skeletal muscle. The results demonstrate that, in contrast to findings on animals, endurance training of humans does not alter the muscle

Table 1. Distribution of nNOS and eNOS in untrained and endurance-trained human skeletal muscle

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<th>Type I Sarcolemma</th>
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nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase. –, Absence of staining; +, faint staining; ++, intermediate staining; ++++, distinct staining.

Fig. 3. Nitrite (open bars) and nitrate (solid bars) concentrations in untrained (Utr) and trained (Tr) muscle at rest and after exercise (ex). Values are means ± SE. dw, Dry weight.

Fig. 4. Neuronal NOS protein content in human muscle biopsy samples obtained from vastus lateralis muscle before (Pre) and after (Post) 6 wk of endurance running training. Values are means ± SE expressed relative to a rat brain standard.
level of nNOS protein or NOS activity. Moreover, training does not appear to alter the qualitative distribution of nNOS in muscle, whereas the number of eNOS containing capillaries is increased.

Training protocols used in previous animal studies, in which an increased level of NOS has been observed, have consisted of mechanical loading, treadmill endurance running, and electrical stimulation performed for periods between 2 days and 8 wk (1, 13, 20). The two 6-wk training protocols in the present study consisted of both intermittent and continuous endurance exercise, and the efficiency of the training was evident by the improvement in endurance performance. The inclusion of two different training protocols in the present study, neither of which resulted in a change in muscle nNOS levels, suggests a species difference, as opposed to choice of training protocols, as the most likely explanation for the discrepancy between the findings of the present study and previous animal studies (1, 13, 20). The reason for the species difference is uncertain but could be related to the fact that, in animals but not in humans, the amount of nNOS present in oxidative muscle fibers is small compared with that found in fast-twitch fibers (9). The oxidative muscle fibers of animals may thus be more responsive to training. Whether the observation also reflects a functional difference for NOS between laboratory animals and humans remains to be elucidated.

Immunohistochemical staining was performed on biopsies taken before and after the one-legged knee-extensor training. Positive nNOS staining was observed both in the sarcolemma and in the cytosol of the muscle fibers, with stronger staining evident in the sarcolemmal region as well as in the cytosol of type I compared with type II fibers. Previous studies have suggested that nNOS in the cytosol colocalizes with mitochondria (6), a proposition that is supported by reports on the presence of NOS in isolated mitochondria (7, 19). The hypothesis behind the present study was that the enhancement in oxidative capacity, including an increased number of mitochondria, associated with endurance training (16) would be paralleled by an elevated amount of nNOS in the cytosol of type I fibers. Although there was evidence for an enhanced oxidative capacity in the trained leg in the present study, as shown by a higher level of citrate synthase, no evidence for a training-induced change in staining intensity or distribution was found for nNOS, either in the cytosol or in the sarcolemma of type I or type II fibers.

As a measure of NO formation in vivo in the control and the trained leg, nitrite and nitrate levels were determined in muscle biopsy samples obtained before and after a submaximal exercise bout performed with the trained and untrained legs. The nitrite and nitrate concentrations at rest in the trained and untrained leg were similar, which agrees with the lack of increase in nNOS levels in muscle with training. The nitrite and nitrate concentrations after exercise were similar to preexercise levels, and they were not different between the untrained and the trained leg. These observations do not support previous findings from animal studies in vitro, which show an increase in nitrite and nitrate in buffer surrounding contracting muscle (1, 20). The reason for this latter apparent discrepancy is unclear but could potentially be explained by the use of different experimental models.

In addition to the analysis of nNOS levels, an immunohistochemical analysis of eNOS was performed that showed eNOS-positive staining in the endothelium of microvessels. eNOS immunoreactivity was not observed in muscle fibers, a finding that contrasts with the results from one of the first reports on the distribution of NOS in rodent muscle that show eNOS in the cytosol in association with mitochondrial density (10).

A more recent study on rat diaphragm muscle has, however, shown that the localization of eNOS is restricted to the vascular endothelial cells (5). The reason for these dissimilarities remains unclear but could potentially be related to the use of different antibodies with differing antigen specificities.

In comparing untrained and trained muscle, the number of eNOS-immunoreactive structures, tentatively identified as microvessels, per muscle fiber was found to be significantly higher in the trained muscle, as was the number of capillaries per muscle fiber. This finding suggests that, when the number of capillaries in the muscle is enhanced by endurance training, the total amount of eNOS in the muscle increases.

In conclusion, the present study shows that endurance training in humans does not alter the protein level, the activity, or the distribution of nNOS in skeletal muscle. Endurance training does, however, increase the number of eNOS-immunoreactive microvessels per muscle fiber in parallel with an increase in the number of capillaries. Moreover, the levels of nitrite and nitrate in untrained muscle are similar to those of endurance-trained muscle and are not altered with submaximal exercise.

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