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ScienceDirect
Biochemical and Biophysical Research Communications 348 (2006) 259–264
BBRC
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Nitric oxide synthase is up-regulated in muscle fibers in muscular dystrophy

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Received 10 July 2006
Available online 20 July 2006

Abstract

Nitric oxide (NO) mediates fundamental physiological actions on skeletal muscle. The neuronal NO synthase isoform (NOS1) was reported to be located exclusively in the sarcolemma. Its loss from the sarcolemma was associated with development of Duchenne muscular dystrophy (DMD). However, new studies evidence that all three NOS isoforms—NOS1, NOS2, and NOS3—are co-expressed in the sarcoplasm both in normal and in DMD skeletal muscles. To address this controversy, we assayed NOS expression in DMD myofibers *in situ* cytophotometrically and found NOS expression in DMD myofibers up-regulated. These results support the hypothesis that NO deficiency with consequent muscle degeneration in DMD results from NO scavenging by superoxides rather than from reduced NOS expression.

Keywords: Nitric oxide synthase; Duchenne muscular dystrophy; Immunohistochemistry; Cytophotometry

In skeletal musculature, the neuronal NOS (nNOS, designated also as NOS1) was originally reported to be localized around the border of some muscle fibers identified as type II (fast) fibers [1]. In the following decade, the circumferential NOS1 immunostaining pattern of muscle fibers, albeit without discriminating between fast and slow myofiber types, was reproduced by various groups. Positive NOS1 immunolabeling [1–8] and NADPH-diaphorase staining [9] surrounding the myofibers were interpreted by the above-quoted authors as a proof for localization of NOS1 in the sarcolemma. The loss of NOS1 from skeletal muscle sarcolemma was declared to underlie the development of the clinical phenotype of DMD [2,3]. This concept cannot be, however, well reconciled with the fact that the correction of some pathology in *mdx* mice (a mouse model of Duchenne myopathy) by dystrophin expression or by utrophin over-expression was independent of the presence of NOS1 [10]. Interestingly, mice lacking NOS1 did not develop a muscular dystrophy [4] in spite of the originally reported association of DMD with the loss of NOS1 from skeletal muscle sarcolemma [2,3].

With the advent of more powerful immunohistochemical technique employing antigen retrieval [11] in combination with signal amplification [12,13], it was shown that NOS1 in skeletal muscles was not restricted to the sarcolemma and co-existed with NOS2 and NOS3 also in the intracellular compartments such as mitochondria, sarcoplasmic reticulum, and along contractile filaments [14].

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.056

Recently, it was reported that, in contrast to the commonly accepted view, skeletal muscles of DMD patients retained all three NOS isoforms [15]. To address this controversy, we have undertaken in this study a quantitative *in situ* determination of NOS expression in muscular fibers of DMD patients using tyramide signal amplification with following cytophotomentrical assessment of absorbance values for NOS1–3 immunostaining. In view of the NO involvement in muscle repair [16,17], a better understanding of the NO pathways in dystrophic skeletal muscles may provide further insight into fundamental mechanisms underlying the development of a clinical phenotype in DMD patients and might have implications in the interception of the NO signaling for designing new adjunctive therapies for muscular dystrophies.

Materials and methods

Muscle biopsies, patients. Muscle biopsies obtained during routine diagnostic procedures from five DMD patients (aged 2–4 years) as well as from neuropathologically normal muscle biopsies ($n = 5$) could be examined. Informed consent for the scientific use of the diagnostic samples was obtained from the patients or their parents. Tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until use or fixed in buffered 4% formaldehyde and routinely embedded in paraffin. The diagnosis of DMD was based on clinical data, laboratory results, and neuropathological findings on biopsy including immunohistochemistry for dystrophins and sarcoglycans. A clinical summary of the patients is given in Table 1.

Immunohistochemistry. Immunostaining of NOS was performed using previously characterized rabbit primary polyclonal antibodies recognizing NOS1, NOS2, NOS3 (Transduction Laboratories, Lexington, KY, USA, and Santa Cruz Biotechnology, Santa Cruz, California, USA) [14,15,18–20]. Four-micron sections of the paraffin blocks were dewaxed in xylene, rehydrated in graded alcohols, and transferred into phosphate-buffered saline (PBS). PBS was used for all washings and dilutions. After antigen retrieval, quenching endogeneous peroxidase activity and blocking non-specific binding sites, sections were immunoreacted with primary antibodies over night at 4 °C as described earlier [15]. Bound rabbit primary antibodies were detected using DAKO anti-rabbit EnVision-HRP (DAKO Corporation, Hamburg, Germany) and NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA), counterstained with Ehrlich hematoxylin for 30 s, and mounted with an aqueous mounting medium GelTol (Immunotech, Marseille, France).

For cytophotometry, frozen tissue probes of DMD and normal muscles were mounted pairwise on a cryostat chuck, and 10 μm cross sections were cut with a cryostat 1800 (Reichert Jung, Vienna, Austria). Mounting and sectioning of DMD and normal muscle probes pairwise on the same glass slide eliminated possible variations in section thickness and incubation conditions that might influence the optical density of immunostained tissue sections. Sections were fixed in cold acetone for 15 min and thoroughly air-dried for 30 min. After rinsing in PBS, nonspecific binding sites were blocked by incubation in PBS containing 10% goat serum for 30 min.

Table 1
Clinical summary of the patients

Patient	Diagnosis	Sex	Age	Clinical symptoms
1	DMD	M	2	Leg weakness, difficulties standing up
2	DMD	M	2	Leg weakness, difficulties standing up
3	DMD	M	3	Impaired motor development
4	DMD	M	4	Leg weakness
5	DMD	M	4	Developing hypotony since age 2

Cryostat sections were incubated overnight at 4 °C with anti-NOS primary antibodies diluted to a final concentration of 1.0 μg ml^{-1}. To quench endogenous peroxidase activity, sections were treated with methanol containing 1.2% H_2O_2 for 15 min. Bound primary antibodies were detected by employing horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies, which was followed by tyramide signal amplification (Biotin-TSA-kit, NEN, Cologne, Germany) and ABC technique using the Vectastain ABC-kit (Vector Laboratories). The visualization was performed using NovaRed substrate kit for peroxidase (Vector Laboratories). The reaction was controlled under the microscope and stopped by rinsing with water after 4–10 min. Control incubations were: (i) omission of primary AB; and (ii) substitution of primary antibodies by rabbit IgG (Dianova, Hamburg, Germany) at the same final concentration as the primary antibodies. The exclusion of either the primary or the secondary antibody from the immunohistochemical reaction, substitution of primary antibodies with the corresponding IgG at the same final concentration, or preabsorption of primary antibodies with corresponding control peptides resulted in lack of immunostaining. The TSA step alone did not contribute to any specific immunostaining that might have influenced the analysis.

Cytophotometry. The absorbance of the NOS-immunostaining of the muscle fiber cryosections was measured with a computer-controlled microscope photometer MPM 200 with a scanning table (Carl Zeiss, Oberkochen, Germany). Absorbance values for immunostained muscle sections of DMD and normal muscles arranged in pairs on the same glass slide were assessed by setting the absorbance of normal muscle fibers at 100%. The difference in absorbance between normal and DMD muscle fibers was taken as a measure of change in NOS immunoreactivity in DMD muscle. Five normal and five DMD muscle sections were analyzed. In each muscle section, more than 30 fibers were separately measured. Statistical analysis was performed by the paired Student's t test using SPSS-software. A level of significance of $p < 0.05$ was considered as sufficient in all experimental groups. Data are means ± SD.

Visualization and image processing. Immunostained paraffin sections were examined on a motorized Zeiss Axiophot2 microscope. Color images were captured using AxioCam 12-bit camera and AxioVision single channel image processing (Carl Zeiss Vision GmbH, Germany). Resulting images were imported as BMP files into PhotoImpact 3.0 (Ulead Systems, Inc. Torrance, CA, USA) for analysis on Power PC followed with printing on a color printer Canon PIXMA iP5000. Images shown are representative of at least five independent experiments which gave similar results.

Western blotting. Skeletal muscle biopsies were homogenized in modified RIPA-buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and complete protease inhibitor cocktail (Roche, Mannheim, Germany)) and incubated for 20 min on ice. After 15 min centrifugation (20000g, 4 °C) protein concentration of supernatant was determined using BCA-kit (Pierce, Rockford, USA). For better separation protein lysates were cleaned using cleanup-kit (GE Healthcare, Amersham, Freiburg, Germany). Proteins (50 μg) were separated in 6% acrylamide gels and transferred overnight with 30 V onto PVDF membranes (trans-blot cell apparatus, Bio-Rad, München, Germany). Transfer onto membrane was checked using fast green stain. After destaining, blots were blocked for 1 h in 5% nonfat skim milk (solved in TBS with 0.1% Tween 20). First antibodies were diluted in blocking solution (NOS1 1:500, NOS2 1:500, NOS3 1:250, all from Transduction Laboratories) and incubated on the blots overnight at 4 °C. Positive controls were for NOS1 rat cerebrum lysate, for NOS2 macrophage +IFNγ/LPS-lysate, and for NOS3 human endothelial cell lysate, all from Transduction Laboratories. Signals were detected by chemiluminescence using ECLplus system (GE Healthcare, Amersham, Freiburg, Germany). Comparability of protein content in the samples used was proven by determination of GAPDH expression (housekeeping protein).

Results and discussion

Skeletal muscle biopsy samples of DMD patients under study revealed typical morphological changes characteristic for this disease, such as a high variability of the muscle

fiber diameter with progressive loss of myofibers accompanied by connective tissue and adipose tissue replacement. Like in healthy muscles [14,20], NOS1–3 isoforms were found to be co-expressed in the same myofibers of DMD as evidenced from examination of adjacent immunostained cross sections. However, in contrast to normal muscles (Fig. 1A), NOS immunoreactivity in DMD muscle fibers (Fig. 1B) varied along the fiber length, where seemingly normal NOS-positive fiber segments intermingled with hypercontracted and with NOS-negative fiber segments. Whereas all three NOS isoforms revealed a pronounced cross-striation immunostaining pattern in the sarcoplasm of muscle fibers in healthy control as shown here for NOS1 (Fig. 1C), NOS-positive segments of DMD muscle fibers revealed a partial loss of cross-striation pattern, disturbed myofilaments architecture, and centrally located nuclei (Fig. 1D). Cross-striation pattern was completely absent in the NOS-negative and in hypercontracted segments of DMD muscle fibers.

No exclusive or preferential NOS1 immunostaining along the myofiber plasma membrane was observed either in normal (Fig. 1C) or in DMD (Fig. 1D) skeletal muscles. We could not see any exclusive or preferential NOS1–3 immunogold staining of sarcolemma also in normal rat skeletal muscles in our previous ultrastructural study [14]. Likewise, we do not observe any preferential NOS1 targeting to the sarcolemma of human skeletal muscles in our current electron microscopical studies (in preparation). Nevertheless, the concept of exclusive NOS1 localization in the sarcolemma is entertained in all current textbooks and reviews on myology and dystrophinopathies. It should be noted that the sarcolemma, which measures only 5–8 nm wide, is much too small to be seen with the light or fluorescent microscope (whose limit of resolution is 0.2 μm). Moreover, the microscopical image of the fluorophore signal or the layer of chromogen deposits surrounding muscle fibers varies from 0.5 to 2.0 μm, and this is a few orders of magnitude above the real thickness of the sarcolemma. Therefore, NOS immunostaining of this unidentified layer [1–8] surrounding muscle fibers can also account for the endomysium [21], as well as for subsarcolemmally clustered mitochondria [14] or caveolae [22], if not for all of them altogether.

In view of the principal disagreement between our recent report evidencing for NOS expression in DMD muscle fibers [15] and reports from other groups about the absence of NOS in dystrophic skeletal muscles [1–8], we have undertaken in this study a quantitative *in situ* determination of NOS expression in muscular fibers of DMD patients. DMD and normal muscle probes were sectioned and mounted pairwise on the same glass slide, which eliminated possible variations in section thickness and incubation conditions that might influence the optical density of immunostained tissue sections. Absorbance values for

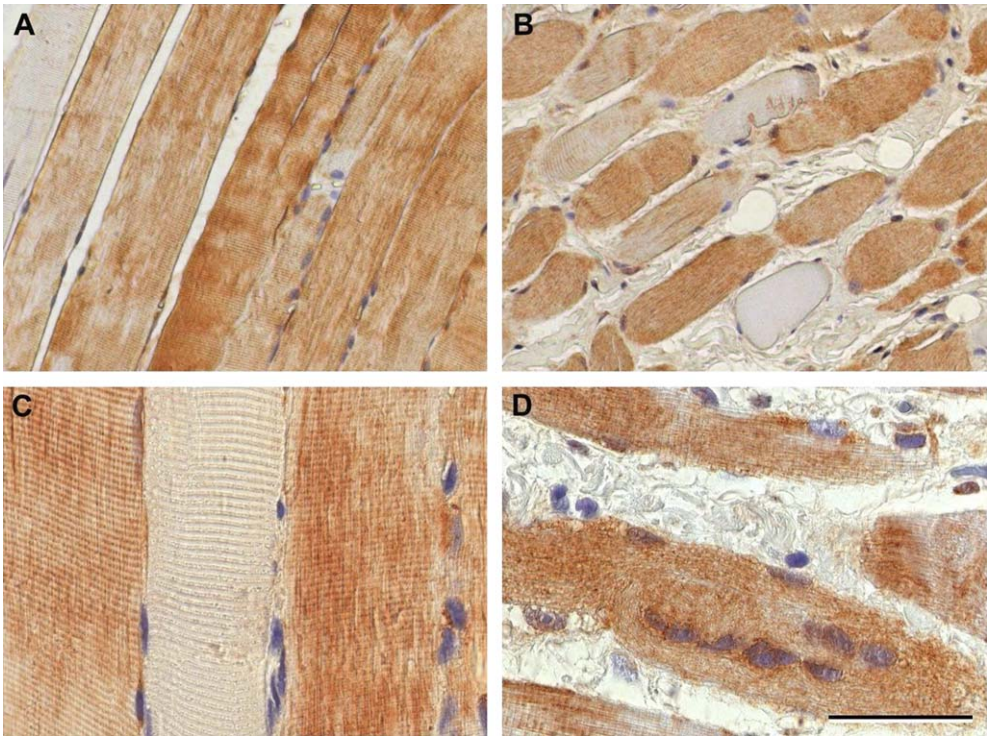

Fig. 1. NOS1 immunoreactivity of skeletal muscle fibers. NOS1 in normal skeletal muscles (A,C) is located along contractile filaments with a pronounced cross-striation pattern. NOS-positive segments of DMD muscle fibers (B,D) do not show any significant decrease in NOS1 immunoreactivity; proliferating connective tissue is NOS negative. Higher magnification permits to demonstrate a progressive loss of cross-striation pattern, disturbed microfilament architecture, and centrally located nuclei (D). No preferential NOS1 immunostaining along the myofiber plasma membrane either in normal (C) or in DMD (D) skeletal muscles. Nuclei are counterstained with Mayer's hematoxylin. Scale bar is 100 μm for (A,B) and 40 μm for (C,D).

immunostained muscle sections of DMD and normal muscles arranged in pairs on the same glass slide were assessed by setting the absorbance of normal muscle fibers at 100%. The difference in absorbance between normal and DMD muscle fibers was taken as a measure of change in NOS immunoreactivity in DMD muscle. Cytophotometrical assessment of absorbance values for NOS1–3 immunostaining showed that NOS1 expression was not down-regulated in muscle fibers of DMD patients, whereas NOS2 and NOS3 expression significantly increased up to 132% and 134%, respectively (Fig. 2).

In contrast to the cytophotometrical assay, Western blotting showed a significant reduction of NOS1 immunoreactivity of DMD skeletal muscles without any clear-cut changes in the immunoreactivity for NOS2 and NOS3 (Fig. 3). This apparent inconsistency between cytophotometrical assay and Western blotting can be explained by a replacement of myofibers by connective and adipose tissues in dystrophic muscles. Indeed, NOS1 immunoreactivity of DMD myofibers, as demonstrated by immunohistochemistry, was comparable to that of healthy control (Fig. 1), whereas connective and adipose tissues were NOS1 negative. An increasing ratio of NOS-negative connective tissue associated with a reduction of NOS-positive muscle tissue (see Fig. 1B and C) inevitably leads to the underestimation of NOS expression in the muscle tissue in DMD muscle tissue probes in Western blotting (Fig. 3).

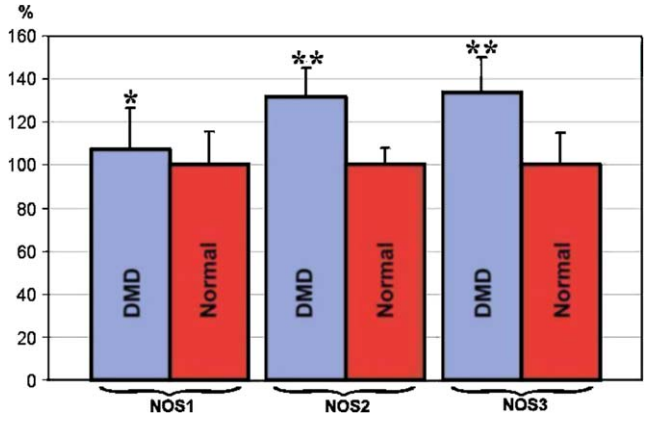

Fig. 2. NOS1–3 expression in DMD muscle fibers vs. healthy control as assayed by cytophotometry. Normal NOS expression was taken as 100%. Data are means ± SD in 60 measurements. *$p > 0.05$, **$p < 0.01$ compared to control samples.

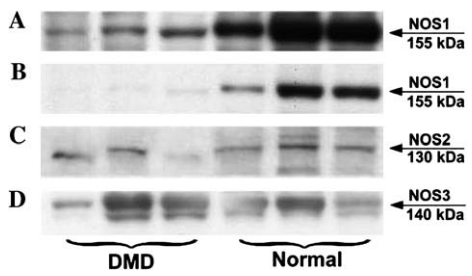

Fig. 3. Western blotting of NOS1 (A,B), NOS2 (C), and NOS3 (D) in muscle probes of DMD patients vs. healthy control

Earlier, we have reported that the expression of NOS1 and NOS3 mRNA in DMD muscles remained practically at the level of healthy control, whereas the expression of NOS2 mRNA significantly increased [15]. This can be well reconciled with results of the present study but is, however, in an obvious conflict with reports from other groups [2–5,7,8] about exclusive NOS1 localization in the sarcolemma and about the loss of NOS1 from DMD muscle fibers. Statements of the above-quoted authors about NOS1 targeting to sarcolemma and about the loss of NOS1 from DMD muscle fibers may be well due to a low detection level in their experimental approaches and to misapprehension of the resolution limit of the light optic. To illustrate how a low detection limit can lead to the false conclusion about NOS1 disappearance from DMD muscle fibers, we are showing here the same NOS1 blot after different screening exposure (Fig. 3A and B). Blot (A) and blot (B) represent NOS1 immunostaining with chemiluminescence exposure times of 1 min and 10 s, respectively. It is evident that the use of inadequate immunohistochemical technology, like a shorter screening exposure, could not allow a detection of NOS1 in DMD myofibers.

As shown in this study, NOS1 expression in DMD skeletal muscles, in contrast to the commonly accepted view, is comparable to that of healthy control whereas NOS2 and NOS3 expression in DMD significantly increases. Up-regulation of NOS2 and NOS3 in skeletal muscles of DMD patients might be an adaptive mechanism aimed at maintaining the homeostatic cellular level of the bioactive NO. This assumption can be well reconciled with reports that L-arginine, the substrate for nitric oxide synthase, and molsidomine, a therapeutic agent that is converted to a NO donor, attenuated the dystrophic phenotype in *mdx* mice [17]. Possibly, some pathology in this animal model was compensated by a total NOS up-regulation. Our findings about NOS2 up-regulation in dystrophic muscles are in accordance with reports about increased NOS2 expression found by other authors in muscle fibers in idiopathic inflammatory myopathies [21] and in DMD patients [10]. These findings are also well in line with indication of a protective role of endogenous NO in inflammatory diseases [23–25] and in muscle repair [16].

Increased NOS protein expression is associated with a higher NO generation (Ref. [26] and citations therein). This poses a question, why total NO levels in DMD patients are nevertheless significantly lower compared with healthy controls [27,28]. Assuming that the reduction of NO levels in DMD patients might be secondary to oxidative stress, we have earlier carried out an immunostaining for nitrotyrosine (footprint of oxidative stress) and found its dramatic up-regulation in dystrophic muscles both in muscular fibers and in vasculature [15]. This is well reconciled with biochemical reports evidencing that dystrophin mutations predict cellular susceptibility to oxidative stress [29]. Enhanced nitrotyrosine immunostaining in muscular fibers as well as

in vasculature of DMD patients may reflect a massive oxidative stress resulting in withdrawing NO from its regular physiological course via the scavenging actions of superoxides. Complemented with results of the present study, these findings imply that chronic reductions in NO bioavailability via the scavenging actions of reactive oxygen species rather than reduced expression of the muscle fiber-associated NOS may be the main, if not the sole, driving force leading to apoptotic cell death mechanisms and muscle fiber loss in muscular dystrophies. Moreover, enhanced nitrotyrosine immunoreactivity in the arterioles in skeletal muscles of DMD patients [15] as well as constitutive local NOS expression in smooth muscle cells of blood vessels [18,19], including vasculature of skeletal muscles [15], imply that impaired muscle perfusion in DMD must be due to chronic reductions in NO bioavailability via the scavenging actions of superoxides [30] rather than to the reduced production of the "sarcolemma-associated" NOS1 as claimed by Sander [8] and Crosbie [5].

To conclude, this study lends evidence for the total NOS up-regulation in skeletal muscle tissue of DMD patients. Taken together with reports that skeletal musculature in DMD undergoes a massive oxidative stress, these findings support the hypothesis [15] that NO deficiency with consequent muscle cell degeneration in muscular dystrophies results from NO scavenging by superoxides rather than from reduced NOS expression. This suggests that pharmacological activators of the NO pathway may constitute a realistic treatment for muscular dystrophies.

Acknowledgments

We thank all colleagues from the immunohistology laboratory for sharing probes and reagents. This study was supported by the DFG Grant SCHM 1398.

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