Does Normal Nitric Oxide Synthase Prevent Pathologic Muscle Changes in Dystrophin Deficiency?

Irena Niebroj-Dobosz(1, 2), Anna Fidziaska(2), Zofia Glinka(1) and Irena Hausmanowa-Petrusewicz(2)

(1) Department of Neurology, Medical University, and (2) Neuromuscular Unit, Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

Abstract

Neuronal nitric-oxide synthase (nNOS) is a member of the dystrophin-associated proteins, regulates homeostasis of reactive free radical species and may contribute to oxidative damage to proteins in muscle diseases. To test the hypothesis that nNOS activity may be involved in sparing from muscle pathology in dystrophin deficient muscles we examined nNOS immunoreactivity in muscles from Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). The results were compared to nNOS in dystrophin positive limb-girdle dystrophy (LGMD) patients. Similar studies in dystrophin deficient hind limb muscles and diaphragm of clinically almost asymptomatic mdx mice were performed. In the DMD patients nNOS appeared to be either drastically reduced, or absent. In BMD and LGMD it was decreased, or normal. In mdx mice muscles no changes in nNOS immunoreactivity were present. In the immunocytochemical examination in DMD nNOS was either not stained, or the staining was observed in the surrounding connective tissue. In BMD nNOS staining was decreased or absent, in LGMD it appeared in the muscle cell cytoplasm. In mdx mice muscles nNOS reactivity was observed on the surface of the muscle fiber, starting from 30 days of age of the animals clusters of nNOS positive cells were observed.

It is suggested that the decrease of nNOS content in dystrophinopathies is contributing to oxidative damage to muscle proteins, which enhances the degeneration of the muscle fibers. Normal nNOS may be one of factors, which prevent pathological muscle changes in mdx mice muscles. Regulations of the activity of this enzyme may be one of the possible strategies in dystrophy treatment.

Key words: dystrophinopathies, mdx mice, nNitric oxide synthase, oxidative damage.

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NitrNitric oxide synthase (nNOS), originally purified from neuronal tissues, has an important level of expression in skeletal muscles [27]. It appeared to be associated with sarcolemma [20]. It is enriched at myotendinous and neuromuscular junctions [41] and binds to dystrophin directly/indirectly via (α'-syntrophin [6, 7, 15, 17, 38]. It is suggested that nNOS is not attached to sarcolemma in Duchenne dystrophy (DMD) [6, 9, 16, 19]. As the consequence of the dystrophin-glycoprotein complex disruption nNOS is downregulated in the cytosol of the muscle cell [6, 16, 17, 19]. nNOS is also absent in Becker dystrophy (BMD) [9, 10, 16] and the mdx mice muscles [5, 6, 9].

As nNOS is implicated in several vital functions in the muscle cell it is of importance to answer the question, whether its deficiency may be responsible, at least partly, for the severe and widespread pathologic changes in the muscles of DMD patients.

Material and Methods

Twenty five patients with DMD (4 to 18 yrs of age), 10 patients with BMD (aged 8 to 20 yrs of age), 11 patients with LGMD (9 to 44 years of age) and 20 aged-matched healthy controls were examined. The diagnosis of the diseases was established by clinical, genetic, electromyographic, histologic, ultrastructural, immunocytochemical, biochemical and immunocytochemical examinations. Diagnostic muscle biopsies were obtained after informed consent of the patients or their families.

Male mutant C57BL/10 mice (14 days to 12 months of age) and control age-matched normal mice of C57BL/6J strain were examined. The animals (5 in each group) were fed a standard laboratory diet. At 14, 30 90
and 365 days of age the extensor digitorum longus (EDL) and diaphragm muscles for immunohistochemical examination, and gastrocnemius and diaphragm for immunochemical examination were taken.

Cryostat frozen sections (8 mm) of the muscles were stained according to the standard techniques. For immunocytochemical studies monoclonal anti-nitric oxidase antibodies (Sigma), diluted 1:3000, were used. Immunostaining: an indirect immunofluorescence method with TRITC immunoglobulins was applied.

For Western blotting examinations human and animals muscles were frozen quickly in dry ice and preserved at -72°C until used. After thawing all procedures were conducted at +4°C. The samples were weighed, minced and homogenized in 50 mM phosphate buffer (pH 7.5) with added protease inhibitors (5 mg leupeptin, 5 mg antipain, 5 mg pepstatin and 170 mg PMSF in 10 ml of DMSO. 100 ml of the mixture per 10 ml of the buffer was used). The concentration of the proteins in the homogenized samples was estimated by the method of Peterson [31]. An aliquot equivalent to 100 µg of proteins was treated with a Laemmli sample buffer. The SDS-polyacrylamide gel electrophoresis in MiniProtean II 4-15% gradient gels (BioRad) in the system of Laemmli [21] in MiniProtean II Electrophoresis Cell was developed. The separated proteins were blotted on nitrocellulose membranes 0.2 µm (LKB) in 25 µM Tris-HCl, 192 mM glycine and 20% methanol buffer (pH 8.3) using Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). The blots were incubated with monoclonal anti-nitric synthase antibodies (Sigma), diluted 1:3000, goat anti-mouse-HRP secondary antibodies, and streptavidin HRP (Opti-4CN detection kit - BioRad). The nitrocellulose sheets were further processed in a GelDoc 1000/2000 system (BioRad), Multi An-alyt/PC version 1.1 and Mitsubishi Video Printer P91.

Figure 1. Quadriceps femoris muscle. (a) Dystrophin-positive muscle fibers in healthy human muscle. (b) nNOS-positive fibers in healthy human muscle. (c) nNOS-negative muscle fibers in DMD. (d) nNOS-decorated endomysial connective tissue in a patient with advanced stage of DMD. x 448

Figure 2. Quadriceps femoris muscle. (a) Early stage of BMD - faint nNOS stained muscle fibers. (b) Advanced stage of BMD - in a small proportion of fibers nNOS is partially stained. (c) LGMD - nNOS stained in the muscle cell cytoplasm. x 448
Analyses of variance, comparison of means and the degree of significance were performed. A single value was defined to be significant when it was over the normal mean ± (SDx2) using the Student’s test and the Wilkoxon’s test. A value over P > 0.05 was considered as non-significant.

Results

In the immunocytochemical studies (ICH) in control human muscle immunoreactivity of nNOS was present in the sarcolemma and closely corresponded to dystrophin. In early stages of DMD no immunoreactivity of nNOS was observed, in more advanced DMD cases the endomysial connective tissue reacted with the anti-nNOS antibodies (Fig. 1). In early stages of BMD very weak nNOS staining in the sarcolemma was present, in more advanced BMD cases nNOS immunoreactivity was more variable. In some fibers positive immunostaining appeared on the muscle cell surface, but some fibers were nNOS negative. In LGMD nNOS was stained in the muscle cell cytoplasm (Fig. 2). In normal mice nNOS was localized at the surface of the muscle fiber. The fluorescence was more distinct in EDL, as compared to the diaphragm (Fig. 3, Fig. 4). In mdx mice nNOS reactivity was present on the surface of the EDL and diaphragm fibers. Starting from 30 days of age an increasing number of cell clusters with intensively
stained sarcoplasm was present (Fig. 3, Fig. 4). In the diaphragm some immunoreactivity in the endomysial connective tissue was also observed (Fig. 4).

In the Western blotting analysis (WB) a nNOS immunoreactive protein at 156 kDa was detected. However both in human as well in mice muscles there were several other immune reactive bands (190 kDa, 170 kDa, 109 kDa, 84 kDa, 45 kDa, 33 kDa and 18 kDa). In DMD muscles the nNOS protein band, was either absent or decreased. In BMD and LGMD nNOS was moderately decreased, or normal (Fig. 5, Table 1). In normal hind limb muscles of non-mature mice (14 days of age) nNOS reactivity was lower than in adult animals, in normal diaphragm the nNOS reactivity was comparable between 14 days and 1 yr of age. Normal nNOS immunoreactivity was present in the mdx hind limb muscles and diaphragm (Fig. 5, Table 1).

Discussion

nNOS is one of the most regulated enzymes in biology. It is responsible for the synthesis of the molecular messenger nitric oxide (NO), which is implicated as a modulator of skeletal muscle contractility and exercise-induced glucose uptake, mitochondrial respiration, carbohydrate metabolism, neuromuscular transmission, muscle development, and blood flow to skeletal muscles [5, 23, 39]. Both the synthesis of NO and the nNOS stability are finely regulated by Ca²⁺/calmodulin interactions [4], and transient changes in intracellular Ca²⁺ [28]. Increases in [Ca²⁺] are required for binding of calmodulin to nNOS and consequently for nNOS activation and its synthesis [28, 32]. nNOS participates also in regulations of the free radical species homeostasis. A progressive increase of nNOS is observed in mice [9] and rats during aging [8], which may indicate a physiological role for NO during the aging process.

Several speculations indicate that decreased nNOS may underlie some aspects of the pathophysiology of dystrophic muscles. nNOS decrease is observed in dystrophin-deficient DMD, BMD muscles [5, 6, 9, 16, 19, 41] and mdx mice hind limb muscles [6, 9]. Downregulation of nNOS is occurring also in dystrophin-positive denervated muscles, neurogenic diseases, metabolic and inflammatory myopathies [16, 33]. nNOS mislocalized to muscle cell cytosol in human dystrophy and in mdx mice is also indicated [11, 12]. It is suggested that cytosolic nNOS could compensate the loss of this enzyme in the sarcolemma [6, 19]. According to other opinions, however, accumulation of nNOS in the cytosol is harmful for the muscle cell, as it may enhance the toxicity of NO or superoxide and contribute to the myofiber necrosis [18].

In all these studies immunocytochemical techniques are used. No immunochemical examination and its quantitation of the nNOS immunoreactive band of 150 kDa is presented, yet. It should be stressed that ICH results should not be directly compared to those of the

![Figure 5. Western blotting of nNOS in human and mice muscles. The position of nNOS is indicated by the arrow.](image)

Table 1. nNitric oxide synthase immunoreactivity in human and mdx mice dystrophinopathy.

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Area of the tracing at 156 kDa mm X 10⁻⁷ OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duchenne dystrophy</td>
<td>25</td>
<td>12.0 ± 11.6* (25)</td>
</tr>
<tr>
<td>Becker dystrophy</td>
<td>8</td>
<td>26.1 ± 13.9* (6)</td>
</tr>
<tr>
<td>Limb-girdle dystrophy</td>
<td>11</td>
<td>40.1 ± 25.8 (7)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>20</td>
<td>66.9 ± 13.3</td>
</tr>
<tr>
<td>Mdx mice - hind limb muscles</td>
<td>20</td>
<td>56.5 ± 14.6</td>
</tr>
<tr>
<td>Mdx mice - diaphragm</td>
<td>20</td>
<td>121.8 ± 35.3</td>
</tr>
<tr>
<td>Normal mice - hind limb muscles</td>
<td>20</td>
<td>60.8 ± 20.9</td>
</tr>
<tr>
<td>Normal mice - diaphragm</td>
<td>20</td>
<td>154.3 ± 64.0</td>
</tr>
</tbody>
</table>

Values are means ± SD. * Significant at P < 0.001. The number of patients with significantly decreased nNOS values (over the mean ± SDx2) is presented in parentheses.
nNOS in dystrophinopathies

As nNOS mediates several functions in the normal muscles loss of this enzyme may underlie some aspects of the pathology in diseased muscles, DMD included. The fact that the most evident nNOS changes were present in dystrophin-deficient DMD/BMD, also in some cases of dystrophin-positive LGMD muscles, but not in the dystrophin-deficient mdx mice muscles, indicates that nNOS decrease is not the consequence of dystrophin deficiency per se. The question remains, what is responsible for the decrease of nNOS in course of muscular dystrophies. One of the possibilities is that nNOS, a protein highly sensitive to calpains, is the subject of the calpains action. The activity of calpains is known to be increased in DMD, at least. This may be one of the mechanisms, which contribute to nNOS changes in DMD [22]. Another mechanism may be connected with the calmodulin-[Ca²⁺]-nNOS interplay. nNOS activity is changed by transient changes of [Ca²⁺], which alters nNOS binding to calmodulin, and consequently induces its activation and synthesis [28, 32]. Both calmodulin [25, 29, 30], and [Ca²⁺] [2, 3, 14, 26, 35, 36] are known to be increased in DMD. This mechanism may contribute to abnormal nNOS activity in DMD.

The question is also, whether nNOS attributes to sparing from dystrophic pathology in dystrophin-deficient but almost clinically asymptomatic mdx mice. This possibility has to be taken into account, but other mechanism(s) in sparing the muscles from dystrophic pathology than is the normal concentration and location of nNOS have to be thought over [40]. Although loss of sarcolemmal nNOS does not seem to be the consequence of dystrophy, the absence/decrease of this enzyme in the muscles may contribute to the oxidative damage to muscle proteins and their increased susceptibility to degeneration. As nNOS is known to increase following exercise [1, 34] and NO regulates blood flow in skeletal muscle [24], playing a major role in muscle tolerance to endurance exercise, the old trials of physiotherapy and exercise in dystrophinopathies should be reminded [13, 37]. Strengthening exercises have rather a harmful effect in DMD, but resisted exercises could have some advantage in management of these patients. The expected exercise induced increase of nNOS activity may improve blood flow and induce a favorable effect on glucose and energy supply to the dystrophic muscle cell.

Address correspondence to:
Irena Niebroj-Dobosz, NW, PhD, Department of Neurology, Medical University, 1a Banacha Str., 02-097 Warsaw, Poland, tel. +48 (22) 659 7505, fax +48 (22) 668 8512.

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References
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