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Nitric oxide synthase (NOS) in mouse skeletal muscle development and differentiated myoblasts

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Abstract The neuronal isoform of nitric oxide synthase (nNOS, termed also NOS-I) is expressed in normal adult skeletal muscle, suggesting important functions for NO in muscle biology. However, the expression and subcellular localization of NOS in muscle development and myoblast differentiation are largely unknown. In the present study, NOS was immunolocalized with isoform-specific antibodies in developing muscle and in differentiated myoblast cultures (mouse C2C12) together with histochemical NADPH-dependent diaphorase activity that is blocked by specific NOS inhibitors and therefore designated as NOS-associated diaphorase activity (NOSaD). Western blot analysis revealed immunoreactive bands for NOS-I-III in lysates from perinatal and adult muscle tissue and C2C12-myotubes that comigrated with prototypical proteins. In embryonic skeletal muscle, but not in adult myofibers, diffuse cytosolic staining and lack of sarcolemmal NOSaD activity and NOS-I immunoreaction were evident. In both myoblasts and fusioned myotubes, NOSaD and NOS isoforms I-III colocalize in the cytosol. Additionally, members of the sarcolemmal dystrophin-glycoprotein complex (i.e., dystrophin, adhalin, β1-dystroglycan) immunolocalize in the cytosol of differentiating myoblasts, whereas anti-dystrophin and anti-β1-dystroglycan clearly delineate the sarcolemma in myotubes. Thus, expression of NOS isoforms I-III and NOSaD is cytosolic in fusion-competent myoblasts during myotube formation in vitro. Interaction of NOSaD/NOS-I with the sarcolemmal dystrophin-complex known from mature myofibers is apparently lacking in prenatal muscle development and differentiating myoblasts. Localization of NOS isoforms thus characterized in myogenic cultures may help further to investigate regulated NO formation in muscle cells in vitro.

Key words NOS isoforms I-III · NADPH diaphorase · Western blot analysis · Dystrophin complex · Muscle development · Myogenic cultures · Mouse

Introduction

The neuronal isoform of nitric oxide synthase (NOS), termed also nNOS, NOS-I, is also expressed in skeletal muscle (Kobzik et al. 1993) at mRNA levels that exceed those in human brain (Nakane et al. 1993). In striated muscle, NO formation appears to be linked to calcium influx, possibly via NMDA receptors, thereby activating NOS (Urazaev et al. 1995), i.e., via the cGMP pathway with guanylyl cyclase, a major molecular target of NO (Kobzik et al. 1994). In cardiac muscle, NO mediates ionotropic and chronotropic depression of heart function via cholinergic vagal activity (Hare et al. 1995). More recently, constitutive expression of endothelial NOS (eNOS, NOS-III) and a cytokine-inducible isofrm (iNOS; NOS-II) has been found in cardiac myocytes (Balgand et al. 1994, 1995). In hippocampal pyramidal neurons NOS-III is coexpressed with NOS-I (Dinerman et al. 1994). Lesion-induced expression of NOS-I protein and mRNA occurs in various types of neurons that normally lack NOS-I (Verge et al. 1992; Wu et al. 1994; Lin et al. 1997), supporting the idea that NOS isoform types I-III can be found in many different types of cells, i.e., neural and non-neuronal cells (Schmidt et al. 1992), and expression patterns are much more complex than previously thought.

In neurons and skeletal muscle cells, NOS-I interacts via PDZ-domains with α-syntrophin (Brenman et al. 1995), a 58 kDa protein member of the plasma membrane-associated dystrophin-glycoprotein complex, thus
determining its unique sarcolemmal positioning in normal adult myofibers (Adams et al. 1993). This prominent molecular association of NOS-I to the sarcolemmal dystrophin-glycoprotein complex is possibly reflected by the “ring-like” immunostaining patterns seen in light microscopy around the cross-sectioned myofibers in adult human skeletal muscle (Kobzik et al. 1993, 1994; Fransen et al. 1996) and many other mammalian muscles (Gath et al. 1995; Grozdanovic et al. 1995). Disruption of the NOS dystrophin complex in mdx mice and human Duchenne muscular dystrophy results in displacement of NOS protein from the sarcolemma to the cytosolic compartment in myofibers—and lack of sarcolemmal immunostaining—most probably associated with the pathophysiology of this severe muscle disease (Brenman et al. 1995).

As known for differentiated neuroblasts (Peuvara and Enikolopov 1995), major functions of NO have also been described for muscle cells in development. NO thus facilitates fusion of cultured single myoblasts to polynucleated myotubes (Lee et al. 1994) and appears to be a retrograde messenger in synaptic signaling in myocyte/neuronal co-cultures (Wang et al. 1995). However, little information on the timing and expression of NOS in development and in myogenic cultures is as yet available.

Here, we show (1) Western blot analysis and immunocytochemistry with antigen-specific antibodies to NOS isoforms (NOS-I-III) and with muscle-specific markers (dystrophin, dystroglycan), (2) NADPH-diaphorase (NOSaD) histochemistry in vitro on single mononucleated myoblasts before and after fusion to polynucleated myotubes, and report on (3) differential localization patterns of NOS-I/NOSaD and dystrophin-complex in myogenic cultures versus developing skeletal muscle in vivo. The differentiating myogenic culture system expressing NOS may help further to elucidate the multifunctional actions of NO and its protective regulation by, for example, relevant biological signals in skeletal muscle development.

### Materials and methods

**Myogenic cultures**

The mouse skeletal muscle cell line C2C12 or rat cell line L8 were obtained from ATCC Inc. (Rockville Md., USA) or kindly provided by H. Jokusch, Bielefeld. Frozen cells were thawed, washed in Dulbecco’s Minimal Essential Medium (DMEM) with 10% fetal calf serum (FCS)/100 U penicillin/100 μg streptomycin/mL, and subcultured for 24 h in tissue culture flasks (Nuncel, Roskilde, Denmark) in culture medium in a 37°C incubator at 89% relative humidity/10% CO₂ atmosphere. Cells were washed in Hanks’ balanced salt solution (HBSS) without Ca²⁺/Mg²⁺, trypsinized (0.05 trypsin/0.02% EDTA, Biochrom KG, Berlin), collected in 20% FCS/DMEM, pelletted, and resuspended in freezing medium (DMEM/10% dimethylsulfoxide). Aliquots were stored in cryotubes (NUNC, Roskilde, Denmark) in liquid nitrogen. For experiments, aliquots of equal passages were thawed, washed with DMEM and cultured at 4×10⁵ cells/25 cm² tissue culture flask in growth medium (DMEM/1 g/l glucose, Biochrom KG, Berlin), including 10% fetal calf serum (FCS)/1% (584.6 mg/l) L-glutamine and 1% non-essential amino acids (NEA)/penicillin-streptomycin (PS) for several days just before confluency of proliferating myoblasts. To induce myotube formation the growth medium was changed to fusion medium (DMEM/5% FCS/5% normal horse serum). Fusion of myoblasts into myotubes was evident in 3–5 days. For histochemistry and immunocytochemistry, myogenic cultures were either grown on 1% aqueous collagen A- (Biochrom KG, Berlin) or on 2% gelatin-coated (Type-B from Sigma Chemicals) coverslips.

**Enzyme histochemistry**

NADPH-diaphorase reaction was performed with myoblast and myocyte cultures essentially according to Scherer and Singer (1983). Briefly, coverslip cultures were thermally washed with HBSS, prefixed in freshly prepared 4% formaldehyde (30 min) and incubated with freshly prepared 2.2 mm β-NADPH (Biomol, Hamburg) in 100 mm Tris-HCl, pH 8.0/0.5% Triton X-100/0.5 mm nitroblue tetrazolium (NBT, Sigma Chemicals) for 1 h at 37°C in the dark. In the histochemical protocol only sterile filtered ultraclean water (Milli-Q UF plus system, Millipore, France) was used, and parallel cultures and muscle tissue sections were stained with the same incubation solutions (i.e., made of identical lots of reduced β-NADPH and NBT) to achieve standardized histochemical stainings. The reaction was stopped by cold phosphate-buffered saline (PBS). Specificity of the NOS-associated NADPH-diaphorase activity (NOSaD) in cultured cells was further controlled by inhibition experiments performed with specific NOS inhibitors using a standardized histochemical protocol (Blottner and Baumgarten 1995).

**Immunological procedures**

**Antibodies**

Polyclonal primary antibodies to C-terminal fragments of the human NOS-I (i.e., a 22.3 kDa fragment corresponding to amino acids aa1095-1289), NOS-II (a 20.4 kDa fragment, aa1030-1209), and NOS-III (a 21 kDa fragment, aa611-1144) NOSc were from Transduction Laboratories, Mamhead, England. Monoclonal antibodies to skeletal muscle proteins, i.e., anti-dystrophin-1 (Dys-1) and -Dys-2, β1-dystroglycan (β1-DG) and adhalin (α1-sarcoglycan) were from Dako (Glostrup, Denmark). Secondary antibodies conjugated with red-fluorescent Cy-3 label were from Jackson Immuno-Research (West Grove, USA).

**Immunocytochemistry**

*Skeletal muscle tissue.* Ten-micrometer-thick cryosections from formaldehyde-fixed (4% in PBS, pH 7.4, freshly prepared) rat embryonic day 19 (E19), postnatal day 5 (P5) (both upper limb muscle and deltoides muscle) and P-90 (adult) skeletal muscle (deltoides) mounted on coated slides were immunostained for 12 h with anti-NOS-I (1:300 in blocking buffer, PBS, pH 7.4/2% serum) in a moist chamber at 4°C, followed by Cy-3 labelled secondary antibody (1:200 in blocking buffer). Control protocols without primary antibody revealed negative staining results (not shown).

![Fig. 1a-f NADPH-diaphorase activity (NOSaD) and NOS-I immunoreactivity in mouse skeletal muscle development (cryosections at longitudinal planes).](image-url)