Neuronal Nitric-oxide Synthase-μ, an Alternatively Spliced Isoform Expressed in Differentiated Skeletal Muscle*

(Received for publication, November 29, 1995, and in revised form, February 13, 1996)

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Nitric oxide (NO) functions as a molecular mediator in numerous processes in cellular development and physiology. Differential expression and regulation of a family of three NO synthase (NOS) gene products help achieve this diversity of action. Previous studies identify post-translational modification and interaction of NOS with specific protein targets as tissue-specific modes of regulation. Here, we show that alternative splicing specifically regulates neuronal NOS (nNOS, type I) in striated muscle. nNOS in skeletal muscle is slightly more massive than nNOS from brain owing to a 102-base pair (34-amino acid) alternatively spliced segment between exons 16 and 17. Following purification, this novel nNOSμ isoform has similar catalytic activity to that of nNOS expressed in cerebellum. nNOSμ appears to function exclusively in differentiated muscle as its expression occurs coincidentally with myotube fusion in culture. An isoform-specific antibody detects nNOSμ protein only in skeletal muscle and heart. This study identifies alternative splicing as a means for tissue-specific regulation of nNOS and reports the first additional protein sequence for a mammalian NOS since the original cloning of the gene family.

Endogenous nitric oxide (NO) formation is catalyzed by a family of NO synthase (NOS) enzymes that directly produce NO from L-arginine and NADPH in a calmodulin-dependent reaction that stoichiometrically produces citrulline as a coproduct (1–5). Molecular analyses identify three genetic loci for NOS. The corresponding protein products have been named according to their original sites of identification. Hence, neuronal NOS (nNOS or type I) occurs at highest densities in brain; endothelial NOS (eNOS or type III) is prominent in endothelial cells; and inducible NOS (iNOS or type II) expression is dynamically up-regulated in cells following immunological stimulation.

Endogenous NO is originally identified as the endothelial derived factor responsible for smooth muscle relaxation (6). More recent studies have identified major functions for NO in the development and physiology of mammalian skeletal and cardiac muscle. NO facilitates fusion of cultured myoblasts (7) and mediates retrograde synaptic signaling in myocyte neuronal co-cultures (8). In mature skeletal muscle, calcium influx associated with muscle depolarization is linked to NO formation which in turn stimulates guanylyl cyclase (9, 10). This cascade modulates contractile force. NO is formed in skeletal muscle by nNOS that is localized beneath the sarcolemma of fast twitch muscle fibers. Subcellular localization is mediated by association of nNOS with the dystrophin glycoprotein complex (11, 12). Disruption of the dystrophin complex in Duchenne muscular dystrophy causes a displacement of nNOS from the sarcolemma to the cytosol. This aberrant localization of nNOS may contribute to disease progression in muscular dystrophy.

Recent studies also indicate a role for NO in cardiac muscle physiology (13). NO activity in cardiac myocytes is stimulated by cholinergic agonists acting at inositol phosphate-linked muscarinic receptors. NO formed in this pathway mediates inotropic and chronotropic depression of heart function in response to cholinergic vagal nerve activity (14, 15). Constitutive expression of eNOS in cardiac myocytes has been described (16).

Excitation-contraction coupling in cardiac and skeletal muscle is a complex process that is modulated by overlapping signaling pathways (17, 18). Enzymes involved in regulation of muscle contraction are often expressed as unique isoforms in skeletal and cardiac muscle. These specialized isoforms may arise from different gene expression (19) or alternative splicing (20). Here, we report that nNOSμ is a novel isoform expressed in skeletal muscle. nNOSμ in muscle is slightly larger than nNOS from brain due to alternative splicing that adds a 102-base pair (34-amino acid) insert between exons 16 and 17. nNOSμ displays catalytic activity similar to that of the nNOS. Expression of nNOSμ is induced coincident with myotube fusion in cultured cells. Throughout the body nNOSμ occurs only in skeletal and cardiac muscle.

EXPERIMENTAL PROCEDURES

RT-PCR and DNA Analysis—Brain, skeletal muscle, and myocyte RNA were isolated using the guanidine isothiocyanate/CsCl method. For RT-PCR, RNA was reverse transcribed with RTh polymerase (Perkin-Elmer) using random hexamer primers according to the manufacturer’s specification (Life Technologies, Inc.). Thirty five rounds of amplification were carried out using the following parameters: annealing, 56 °C, 40 s; extension, 72 °C, 60 s; denaturation, 94 °C, 40 s. The sequences of the PCR primers used were: P1, GTC TTC CAC CAG GAG ATG (sense); P2, AAA GGC ACA GAA GTG GGA GTA (antisense). PCR products were separated on a 1% agarose gel and either excised and subcloned or transferred to a nylon membrane. Hybridization used a random primer 32P probe generated using the full-length (507-bp) nNOS CDNA (21) as a template. The filter was washed at high stringency, 68 °C, 0.1% SSC, 0.1% SDS, and exposed to x-ray film.

Partial Purification of NOS—Homogenates of brain, skeletal muscle, or transfected COS cells were prepared in buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA. For purification, 5 mg of crude protein were incubated with 100 μl of 2.5 ADP-agarose (Sigma); columns were washed with 5 ml of buffer containing 0.25 mM NaCl.
and were eluted with 0.5 ml of 10 mM NADPH.

Measurement of NOS Activity—Purified nNOS was assayed for catalytic activity by monitoring the conversion of \([3H]\)arginine to \([3H]\)citrulline (22). Assays were carried out in 125-μl reactions containing 100,000 cpm \([3H]\)arginine (60 Ci mmol⁻¹), 1 mM NADPH, 3 mM each of tetrahydrobiopterin, FAD, and FMN. Concentrations of calmodulin, free calcium, and labeled arginine were added as described in the experiments. After incubation for 5 min at 22 °C, assays were terminated with 4 ml of H₂O. Samples were applied to 0.5 ml of Dowex AG-50WX-8 (Na⁺-form) columns. \([3H]\)Citrulline was quantified by liquid scintillation spectroscopy of the 4-ml flow-through.

Cell Culture and Transfection—Neuronal NOS cDNAs were cloned into the mammalian expression vector pcDNA 3 (Invitrogen). Monkey COS cells were grown and transfected using calcium phosphate as described previously (21). Primary rat myocytes were cultured by a standard technique (23). Briefly, muscle from fore and hindlimbs of neonatal rats were enzymatically dissociated and plated onto laminin-coated dishes at 10⁵ cells cm⁻². Cells were grown in Ham's F-10 nutrient mixture (Life Technologies, Inc.) supplemented with 20% fetal bovine serum and 2.5 ng/ml bovine fibroblast growth factor (Promega). Myoblasts were fused in media containing Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 2% horse serum.

Generation of nNOS\(_m\) Antiserum—An N-terminally acetylated 18-amino acid peptide (PEPLRFFPRKGPSLHSC), corresponding to a unique sequence of the nNOS\(_m\) alternative splice with a C-terminal cysteine, was synthesized and coupled with \(m\)-maleimidobenzoyl-N-hydroxysuccinimide to keyhole limpet hemocyanin. Rabbis were immunized with the coupled antigen emulsified in Freund's adjuvant. Serum bleeds were evaluated by enzyme-linked immunosorbent assay.

**RESULTS**

NOS activity in mouse skeletal muscle largely derives from the nNOS locus as mutant mice carrying a targeted disruption of nNOS lack detectable NOS activity in skeletal muscle homogenates (11). Western blotting demonstrates that nNOS migrates as a slightly larger protein than nNOS from brain (B). Solubilized protein homogenates from brain and skeletal muscle were partially purified by 2.5% ADP-agarose chromatography, analyzed by 7.5% SDS-PAGE, and probed with nNOS monoclonal antibody. Molecular weight standards are marked at the left side of the panel. B and C, RT-PCR analysis indicates that nNOS mRNA in skeletal muscle contains an additional 102-nucleotide sequence encoding 34 amino acids between Lys-839 and Ser-840 of nNOS. B, products from brain and muscle RNA were amplified using primers P1 and P2, transferred to a nitrocellulose membrane, and the blot was hybridized with a cDNA probe to nNOS. The product from brain migrates as a ~650-bp band whereas that from muscle runs at ~750 bp. C, schematic model showing the nucleotide and predicted amino acid sequence of the unique region amplified from skeletal muscle cDNA. Alignment with eNOS and iNOS shows that the alternatively spliced region corresponds to the domain not present in iNOS. Abbreviations: CaM, calmodulin; myr, myristoylation site.
acids 690–896 of nNOS (21) consistently yielded an nNOS PCR product from muscle that was ~100 bp larger than that from brain (Fig. 1B). All other nNOS primer pairs amplified products of similar size from brain and muscle cDNA (data not shown). Cloning and sequencing of the unique product amplified from skeletal muscle indicated that it contains the appropriate nucleotide sequence of nNOS with the addition of a 102-bp sequence inserted precisely between exons 16 and 17 (24). This 102-base pair insert appears to reside within the 16th exon of nNOS as the newly introduced sequence does not correspond to the sequences of the 5' donor or 3' acceptor sequences in intron 16 of the nNOS gene previously reported (24). This insert occurs after amino acid 839 of nNOS (21) and codes for 34 amino acids that do not share significant sequence homology with NOS or any other cloned gene (Fig. 1C). Adding this novel sequence to nNOS predicts a 164-kDa protein product in skeletal muscle, consistent with that observed by Western blotting. We therefore name this muscle-specific isoform nNOSμ.

We evaluated nNOSμ protein expression by heterologous cell transfection. Expression constructs encoding nNOS or nNOSμ were transfected into COS cells, and following 3 days incubation, protein homogenates from the cells were analyzed. Western blotting demonstrated that nNOSμ migrates as a 164-kDa band that is slightly larger than nNOS which is 160-kDa. The migration of transfected nNOSμ and nNOS is indistinguishable from that of nNOS proteins purified from skeletal muscle and brain, respectively (Fig. 2A).

We evaluated catalytic activity of nNOSμ purified from transfected cells. nNOSμ displayed specific NOS activity similar to that of nNOS. Kinetic constants Vmax and Km (~3 μM) are essentially identical between nNOSμ and nNOS (Fig. 2B). The alternative splice occurs in the vicinity of the calmodulin binding domain of nNOS. We therefore carefully evaluated calcium and calmodulin dependence of the novel isoform. nNOSμ showed an absolute dependence on calcium and cal-
modulin. The EC_{50} for both calcium \((-10 \text{ nm})\) and calmodulin \((-10 \text{ nm})\) was essentially identical for nNOS\(_{\mu}\) and nNOS (Fig. 2, C and D).

Many specialized proteins involved in regulation of contraction are uniquely expressed in differentiated muscle but not in developing myoblasts (25). To determine whether nNOS\(_{\mu}\) represents a regulator of this type, we evaluated expression in cultured myocytes. Primary rat muscle cultures were prepared from postnatal day 3 gastrocnemius. Myoblasts were grown for 48 h in vitro (myoblasts) and fused in differentiation media for 24 h (myotubes). Protein extracts were separated by SDS-PAGE and analyzed by Western blotting. B, nNOS\(_{\mu}\) mRNA expression is induced during myotube differentiation. RT-PCR analysis with primers 1 and 2 shows that amplification from myoblast cDNA generates only a \(-650\)-bp product that corresponds to the nNOS amplified from brain (B). Amplification from myotube cDNA yields a mixture of this \(-650\)-bp product and a \(-750\)-bp product. This larger band comigrates with nNOS\(_{\mu}\) amplified from skeletal muscle (M). + represents amplification from a positive control nNOS cDNA in pBluescript. RNA from cultured cells or rat tissues were processed by RT-PCR; the amplified products were resolved by 1\% agarose gel electrophoresis, transferred to nylon membranes, and the blot hybridized with a labeled nNOS probe.

FIG. 3. Expression of nNOS in primary myocyte culture. A, Western blot shows the appearance of nNOS\(_{\mu}\) (164 kDa) only in differentiated myotubes, whereas the nNOS form (160 kDa) is present in both myoblasts and myotubes. Primary cultures of neonatal rat muscle were grown for 48 h in vitro (myoblasts) and fused in differentiation media for 24 h (myotubes). Protein extracts were separated by SDS-PAGE and analyzed by Western blotting. B, nNOS\(_{\mu}\) mRNA expression is induced during myotube differentiation. RT-PCR analysis with primers 1 and 2 shows that amplification from myoblast cDNA generates only a \(-650\)-bp product that corresponds to the nNOS amplified from brain (B). Amplification from myotube cDNA yields a mixture of this \(-650\)-bp product and a \(-750\)-bp product. This larger band comigrates with nNOS\(_{\mu}\) amplified from skeletal muscle (M). + represents amplification from a positive control nNOS cDNA in pBluescript. RNA from cultured cells or rat tissues were processed by RT-PCR; the amplified products were resolved by 1\% agarose gel electrophoresis, transferred to nylon membranes, and the blot hybridized with a labeled nNOS probe.

To definitively determine whether the alternative splice identified here is translated in vivo, we generated a polyclonal antiserum to a unique peptide sequence encoded by the 102-bp insert (see “Experimental Procedures”). We evaluated specificity of this serum by Western blotting. nNOS and nNOS\(_{\mu}\) from transfected cells and purified nNOS from brain and muscle were resolved by SDS-PAGE, and proteins were transferred to a nylon membrane. The blot was first analyzed with a monoclonal antibody to nNOS that reacted with appropriate 160- or 164-kDa bands in all four lanes (Fig. 4B). We reprobed the same blot with a general nNOS antibody shows selective expression of this isoform in skeletal muscle and heart. NOS proteins from 5 mg of solubilized protein from rat tissues were partially purified by 2'5'ADP-agarose and analyzed by Western blotting. The brain sample was loaded at 10% the level of other tissues to compensate for the higher expression of nNOS in brain.

From nNOS to nNOS\(_{\mu}\) occurs coincident with myotube fusion.

We used this specific antibody to determine the distribution of nNOS\(_{\mu}\) in a variety of tissues. NOS proteins were partially purified from brain, skeletal muscle, lung, liver, heart, and kidney and were separated by SDS-PAGE. Western blotting showed that nNOS\(_{\mu}\) was most prominently expressed in brain...
and, in descending order of abundance, occurs in skeletal muscle, spleen, heart, kidney, and liver (Fig. 4C). By contrast, nNOS\(_\mu\) occurred selectively in skeletal muscle and heart.

**DISCUSSION**

nNOS was first cloned from a brain cDNA library based on peptides sequenced from the purified cerebellar protein (21). Subsequent studies have identified putative alternative splicing of nNOS mRNA. Previously identified examples of alternative splicing within the coding region of nNOS involve deletions of specific exons that are predicted to yield inactive proteins (24, 26). Translation of these alternatively spliced products in intact tissues has not been detected. Alternative splicing of exon 1 of nNOS has been elegantly demonstrated; however, this occurs in the 5'-untranslated domain of the mRNA and does not affect the nNOS protein product (27). The 34-amino acid insert reported here represents the first novel translated sequence for nNOS identified since the original cloning.

The alternative splice we find in muscle nNOS\(_\mu\) occurs between amino acids 839 and 840 of the neuronal protein. Alignment of the three known mammalian NOS gene products shows that they share >50% sequence identity. A significant gap in their alignment occurs only in the region between amino acids 832 and 875 of nNOS. It is interesting to note that the alternative splice we have identified occurs in this particular domain that is divergent from the other NOS isoforms. This region of NOS may be unique in allowing catalytically active alternatively spliced isoforms. Indeed, nNOS\(_\mu\) displays specific catalytic activity similar to that of nNOS. Although the alternatively spliced insert occurs in proximity to the calmodulin binding site of nNOS (amino acids 726–747), nNOS\(_\mu\) displays regulation by calcium and calmodulin similar to that of nNOS. The 34-amino insert contains 5 serine residues in proximity to basic amino acids consistent with possible sites of protein phosphorylation.

NO functions as a physiological regulator of skeletal muscle contractility (9). Formation of NO is coupled to sarcolemmal depolarization (10) owing to the association of nNOS\(_\mu\) with the sarcolemmal dystrophin-associated glycoprotein complex (11). Previous studies show that an N-terminal PDZ/GLGF protein motif is necessary for interaction of skeletal muscle nNOS with the sarcolemma. Disruption of the dystrophin complex in mdx mice and humans with Duchenne muscular dystrophy causes a displacement of nNOS from sarcolemma and accumulation in the cytosol (11). By contrast membrane association of nNOS in brain does not require dystrophin. The selective expression of nNOS\(_\mu\) in skeletal and cardiac muscles, tissues enriched with dystrophin (28), may indicate a role for nNOS\(_\mu\) as an effector enzyme for the dystrophin complex.

In cardiac muscle, NO formation is stimulated by parasympathetic nerve activity (13, 15). NOS inhibitors block the suppression of cardiac muscle contraction that normally occurs following vagal nerve stimulation. Mechanistically, NO appears to inhibit adrenergic increases in L-type calcium currents (14). Molecular studies by Michel and colleagues (16) have demonstrated eNOS expression in protein homogenates from heart. Immunohistochemical studies identified eNOS in capillary endothelial cells of intact heart and in cultured cardiac myocytes. These investigators did not identify iNOS in heart. By contrast we detect low levels of nNOS by Western blotting of protein extracts from rat heart. Furthermore, we detect the nNOS isoform, which is otherwise restricted to skeletal muscle. This likely indicates that nNOS in heart occurs in the cardiomyocytes rather than in neurons or other cell types.

Acknowledgment—We thank Steve Hardy for valuable advice on cloning strategies.

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J. Biol. Chem. 1996, 271:11204-11208.
doi: 10.1074/jbc.271.19.11204

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