Neuronal Nitric Oxide Synthase Isoforms α and μ Are Closely Related Calpain-Sensitive Proteins

ROMUALD LAINÉ and PAUL R. ORTIZ DE MONTELLANO

Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446

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ABSTRACT

The neuronal nitric oxide synthase isoform nNOSμ, which is expressed in striated muscle, differs from nNOSα, the major brain isoform, by the insertion of 34 amino acid residues between the calmodulin- and flavin-binding domains [*J Biol Chem* **271:**11204–11208 (1996)]. We show here that recombinant, purified nNOSμ, despite the peptide insertion, has the same spectroscopic properties, L-arginine k_{cat} and K_m values, optimal pH, and calmodulin binding affinity constant as nNOSα. However, nNOSμ consumes NADPH and reduces cytochrome c at approximately half the rate of nNOSα. The rates of degradation of the two proteins by rat brain and muscle homogenates show that nNOSμ is degraded more slowly than nNOSα. The *in vitro* half-lives of nNOSα and nNOSμ are 12 and 50 min, respectively, and calpain is important for this degradation. These short *in vitro* half-lives suggest that the nNOS isoforms are susceptible to rapid degradation in vivo. The elevated (20-fold) levels of calpain in diseased muscle tissue in Duchenne muscular dystrophy, and the hydrolytic sensitivity of both nNOSμ and nNOSα to this enzyme, may contribute to the deficiency of nNOS activity in the diseased tissue.

NOS enzymes catalyze the NADPH- and O_2-dependent conversion of L-Arg to nitric oxide (·NO) and citrulline (Iyengar *et al.*, 1987; Kwon *et al.*, 1990). This transformation involves two successive oxidations of the L-Arg guanidino function: the first yields N^ω-hydroxy-L-Arg and the second converts this intermediate into the final reaction products (Stuehr *et al.*, 1991). The NOS isoforms are large, multidomain polypeptides that are catalytically active as homodimers (Schmidt *et al.*, 1991). Each of the subunits of the dimer binds heme (iron protoporphyrin IX regardless of the valence and ligation states), FAD, FMN and tetrahydrobiopterin (H_4B) as prosthetic groups. The heme is incorporated into a hemoprotein domain in which a cysteine residue provides the fifth ligand to the iron atom (McMillan *et al.*, 1992; Stuehr and Ikeda-Saito, 1992; White and Marletta, 1992), and the two flavins are part of a reductase domain with strong sequence similarity to cytochrome P450 reductase (Bredt *et al.*, 1991). H_4B binds to the heme domain but its role remains obscure (Mayer and Werner, 1995). In addition to the above cofactors, the oxidation of L-Arg to ·NO and citrulline requires the binding of CaM to a CaM-binding motif connecting the heme and flavin domains (Bredt and Snyder, 1990; Zhang and Vogel, 1994).

The products of at least three distinct NOS genes have been identified in mammals. The neuronal (nNOS, NOS-I) and endothelial (eNOS, NOS-III) isoforms are constitutively expressed in the brain and endothelial cells, respectively (Bredt and Snyder, 1990; Pollock *et al.*, 1991). The activities of these two isoforms are regulated by the Ca^{2+}-concentration because they bind CaM in a Ca^{2+}-dependent manner. The macrophage (iNOS, NOS-II) isoform is not constitutively expressed but its expression is induced by cytokines (Hevel *et al.*, 1991). CaM is tightly bound to iNOS in an essentially Ca^{2+}-independent manner (Cho *et al.*, 1992), so the enzyme activity is controlled by its rate of expression rather than by the Ca^{2+}-concentration.

The neuronal enzyme exists in different isoforms because of alternative splicing of the nNOS mRNA. The principal isoform in brain is nNOSα (Eliasson *et al.*, 1997), but studies of mice with targeted deletions of exon 2 have led to the demonstration that two minor isoforms, nNOSβ and nNOSγ, that do not contain exon 2 account for ~5% of the NOS activity in the brain (Huang *et al.*, 1993; Brenman *et al.*, 1996; Eliasson *et al.*, 1997). Another isoform, nNOSμ, was recently found to be expressed as the single nNOS isoform in rat skeletal muscle (Magee *et al.*, 1996; Silvagno *et al.*, 1996). This novel isoform is slightly larger than the neuronal nNOSα because of an alternative splicing that inserts a 34 amino acid peptide into the protein sequence between the CaM- and FMN-binding domains of the protein. Preliminary

ABBREVIATIONS: NOS, nitric oxide synthase; H_4B, (6*R*)-5,6,7,8-tetrahydrobiopterin; L-Arg, L-arginine; CaM, calcium-dependent calmodulin; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACES, *N*-[2-acetamido]-2-aminoethanesulfonic acid.

studies with partially purified enzyme from COS cells heterologously expressing nNOSµ suggest that the kinetics and CaM binding affinities of nNOSµ are similar to those of nNOSα (Silvagno *et al.*, 1996).

We report here heterologous expression of nNOSµ in *Escherichia coli* and purification and characterization of the protein. The results establish that the 34-amino-acid insert has measurable but small effects on the *in vitro* catalytic activities of the enzyme. Comparison of the degradation of recombinant nNOSα and nNOSµ in incubations with brain and muscle homogenates shows that nNOSα is degraded somewhat more rapidly than nNOSµ. More importantly, these studies reveal that both proteins have very short half-lives because of rapid digestion by calpain, a phenomenon that may be relevant to the Ca^{2+}-dependent regulation of NOS activity and its defect in Duchenne muscular dystrophy.

Experimental Procedures

Materials. The expression and purification of nNOSα was carried out as previously reported (Gerber and Ortiz de Montellano, 1995). Human CaM was expressed in *E. coli* and was purified by phenyl Sepharose (Pharmacia, Piscataway, NJ) chromatography as previously reported (Rhyner *et al.*, 1992). Aurintricarboxylic acid and L-Arg were from Aldrich (Milwaukee, WI), calpain and the calpain inhibitor peptide from Sigma (St. Louis, MO), and H_4B was from Alexis Biochemicals (San Diego, CA). L-[^{3}H]Arg was obtained from Amersham (Arlington Heights, IL). Bradford protein assay kits were from Bio-Rad (Hercules, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). DNA purification kits were purchased from QIAGEN (Chatsworth, CA).

Expression of nNOSµ in *E. coli*. Previous studies in this laboratory have demonstrated that the pCWori vector can be used to express catalytically active nNOSα in *E. coli* (Gerber and Ortiz de Montellano, 1995). The nNOSµ cDNA was provided by David Bredt and Houhui Xia (University of California, San Francisco, CA) (Silvagno *et al.*, 1996). Because nNOSµ originates from an alternative splicing of the nNOS mRNA, the insert coding for the 34 amino acids is found in a *BlpI-BsmI* fragment of the nNOSµ cDNA that is absent in the equivalent *BlpI-BsmI* fragment of the nNOSα cDNA. The *BlpI-BsmI* fragment of the nNOSµ cDNA was therefore incorporated into the pCWori-nNOS plasmid from which the endogenous *BlpI-BsmI* fragment had been excised. Expression and purification of nNOSµ was carried out exactly as described previously for the preparation of nNOSα (Gerber and Ortiz de Montellano, 1995).

Enzyme assays. The activities of the protein preparations were determined by measuring either the production of ˙NO, using the conversion of HbO_2 to Met-Hb, or the conversion of L-[^{3}H]Arg to L-[^{3}H]citrulline at 25° (Hevel and Marletta, 1994). The reduction of cytochrome *c* by the two nNOS forms was assayed at 25° in a 500-µl volume using a previously described method (Rodriguez-Crespo *et al.*, 1996). When indicated, the assay contained CaM in a CaM/NOS molar ratio of three. NADPH oxidation was quantified spectrophotometrically at 340 nm using the extinction coefficient ϵ_{340} = 6200 M^{-1} cm^{-1}. The cuvettes held 500 µl of 50 mM HEPES buffer, pH 7.4, containing 2.5% glycerol, 100 µM NADPH, 10 µM H_4B, 200 µM dithiothreitol, 63 units of catalase, 1 mM Ca^{2+}, 5 µM FAD, and 5 µM FMN. For each rate determination, five different NOS concentrations were used and the CaM concentration was varied to preserve the CaM/NOS ratio of three.

Incubation with cell homogenate fractions. A 0.5-g sample of Sprague-Dawley rat brain or leg muscle was homogenized in 5 ml of 25 mM Tris buffer, pH 7. The homogenate was centrifuged 10 min at 15,000 × *g*. The supernatant and the pellet were separated and the pellet was resuspended in 5 ml of the same Tris buffer. To 5 ml of supernatant or resuspended pellet were added 2 mM ATP (final

concentration), 10 mM phosphocreatine, 40 µg/ml phosphocreatine kinase, 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Incubations were carried out at 25°. The background activities in the supernatant and the resuspended pellet were determined immediately after these additions and 45 min later. The supernatant and the resuspended pellet were then separated into two equal volumes of ~2 ml each and 125 pmol of either nNOSα or nNOSµ was added to both the supernatant and the resuspended pellet.

In the experiments with the inhibitors, aurintricarboxylic acid was added at a 500 µM final concentration and the peptidic calpain inhibitor at a concentration of 50 µg/µl after the 45 min preincubation. In incubations with the calpain inhibitor, aurintricarboxylic acid (Posner *et al.*, 1995), no $CaCl_2$ was added, but $CaCl_2$ was present in incubations with the peptidic calpain inhibitor (Maki *et al.*, 1989).

Determination of the activity in the cell homogenate. At different time points, 250 µl of the solutions from the incubation mixtures above were added to 250 µl of the L-[^{3}H]Arg assay reaction mixture described elsewhere (Hevel and Marletta, 1994). The slope in cpm/min was used to calculate the rate of the NOS activity.

pH dependence of the activity. For this experiment, the usual HEPES buffer was replaced by a buffer mixture composed of 50 mM ACES buffer, 26 mM Tris, and 26 mM ethanolamine (Ellis and Morrison, 1982). This mixture of three components has the advantage of maintaining a constant ionic strength over a wide pH range. The pH was adjusted with either HCl or NaOH to the desired value.

Calpain digestion. The calpain used in this study was the 80-kDa unit of rabbit skeletal muscle m-calpain purchased from Sigma. For these studies, a 50-µl final volume of 50 mM Tris buffer, pH 7.5, containing 90 pmol of nNOSα, 2 pmol of calpain (or none in control experiments), and 2 mM $CaCl_2$ was used. The digestion, carried out at 37°, was stopped at different time points by immersion in boiling water for 1 min. Electrophoresis was done on 8% polyacrylamide gels with a tricine buffer. Western blotting was done using a monoclonal antibody raised against the nNOS reductase domain (Transduction Laboratories, Lexington, KY).

Calpain digestion of endogenous nNOS in brain homogenate. A 0.5-g sample of brain was homogenized in 5 ml of 25 mM Tris buffer, pH 7. To this mixture were added 2 mM ATP (final concentration), 10 mM phosphocreatine, 40 µg/ml phosphocreatine kinase, and 10 mM $MgCl_2$. This mixture was incubated at 25° in the presence or absence of 50 µg/µl of peptidic calpain inhibitor. At different time points, a 500-µl aliquot of each of the incubation mixtures was taken and was boiled. In the absence of the peptidic calpain inhibitor a 1/50 dilution of the boiled aliquot was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. In the presence of the inhibitor, a 1/10 dilution was used. Western blotting was done with a monoclonal antibody raised against the nNOS reductase domain (Transduction Laboratories, Lexington, KY).

Results

Expression and Spectroscopic Characterization of nNOSµ

The spectra of the purified recombinant ferric nNOSα and nNOSµ isoforms are essentially identical (not shown). In the absence of L-Arg, both forms of the enzyme have an absorbance maximum at 400 nm. The addition of L-Arg to both enzymes causes a shift of the absorbance maximum from 400 to 394 nm. These results are in agreement with the expected shift in the equilibrium from low spin to high spin (McMillan and Masters, 1993).

In Vitro Activity

The insertion of 34 amino acids into the middle of the protein sequence might be expected to influence a number of structural and functional parameters, including the catalytic

activity, the absorption spectroscopic properties, the substrate affinity, the degree of coupling of L-Arg oxidation to NADPH consumption, the binding of CaM, and the rate of electron transfer from the flavins to the heme group. We have, therefore, compared the behavior of the two isoforms with respect to these parameters.

CaM binding. Because the peptide insert is close to the CaM binding site, the additional 34 amino acids in nNOSμ might alter its interaction with CaM. To examine this possibility, the rate of oxidation of L-[^{3}H]Arg to citrulline at a constant concentration of the substrate (15 μM) and NOS (7.5 nM) was monitored as the CaM concentration was varied. These measurements provide a pseudo-half saturation constant $K_{1/2}$. This constant depends on the nNOS concentration, but if the concentrations of the two nNOS isoforms are the same, a comparison between them is possible. Very similar binding affinities are obtained for the two enzymes: $K_{\text{nNOSμ}} = 5 \pm 2$ nM and $K_{\text{nNOSα}} = 7 \pm 3$ nM (Table 1). These values are comparable to the value of ~3 nM obtained with nNOSμ expressed in COS cells (Silvagno *et al.*, 1996).

Coupling of the reaction. It has been shown that the reaction of nNOS is uncoupled and that consumption of NADPH in the absence of L-Arg is larger than in its presence (Abu-Soud *et al.*, 1994). As the insert is located near the FMN-binding domain, it might alter the degree of coupling of the reaction by, for example, decreasing the consumption of NADPH in the absence of L-Arg. The NADPH consumption of the two isoforms with and without L-Arg was therefore compared (Table 1). Both enzymes consume NADPH in the absence of L-Arg, but the rate of NADPH consumption by nNOSμ is approximately half that of nNOSα (Table 1). The addition of L-Arg has a similar effect on the two nNOS isoforms in that the NADPH consumption decreases in both cases to 66% of the value in the absence of L-Arg.

Reduction of cytochrome c. Because of the proximity of the FMN binding site to the 34 amino acid insert, it is conceivable that intermolecular electron transfer might be altered. Because CaM is important for this transfer, we measured the rate of cytochrome *c* reduction in the absence and presence of a saturating concentration of CaM (Table 1). Again, the peptide insert has only a modest influence on this parameter. The rate of reduction of cytochrome *c* by nNOSμ is roughly half that for nNOSα, both in the presence and absence of CaM.

TABLE 1
Kinetic properties of nNOSα and nNOSμ

In all experiments, saturating concentrations of H$_4$B and NADPH have been used. The data represent the average of at least two independent experiments done in duplicate. The k_{cat} and K_m determinations were done with saturating concentrations of CaM with the conversion of L-[^{3}H]Arg to citrulline as the assay. $K_{1/2}$ is a pseudo half saturation constant. A fixed concentration of nNOSα or nNOSμ (7.5 nM) has been used with different CaM concentrations. $K_{1/2}$ represents the concentration of CaM required to reach half of the maximum velocity.

Parameter	Additional conditions	nNOSα	nNOSμ
k_{cat} (min^{-1})		30 ± 1	28 ± 1
K_m (μM)		1 ± 0.1	1.2 ± 0.2
CaM binding, $K_{1/2}$ (nM)		7 ± 3	5 ± 2
NADPH consumption^a (min^{-1})	−L-Arg	350 ± 50	150 ± 30
	+L-Arg	230 ± 20	100 ± 10
Cytochrome *c* reduction^b (min^{-1})	−CaM	200 ± 50	100 ± 40
	+CaM	7000 ± 900	2500 ± 500

^{a} Saturating concentrations of CaM and L-Arg (when present) were used.
^{b} When present, a saturating CaM concentration was used.

pH dependence of the activity. Although the differences between the two isoforms in the parameters that would seem to be most susceptible to alteration by the peptide insert are relatively small, it is possible that the two isoforms are located in different subcellular compartments. Because various organelles have different internal pH values, it is possible that the two nNOS isoforms have different optimal pH values. We have therefore measured the catalytic rates of the two isoforms at different pH values with a saturating L-Arg concentration (15 μM) at a fixed CaM/NOS molar ratio of three. Fig. 1 shows the pH dependence of the NOS activity, which is low at pH 5 and pH 8. The optimal pH of approximately 6.4 is similar for both NOS isoforms. The kinetic results summarized here were obtained with the oxyhemoglobin assay, but similar experiments in which the conversion of radiolabeled L-Arg to citrulline was assayed yield essentially identical results. The two enzymes thus have the same pH-activity profile.

Activity in the Supernatant and Resuspended Pellet

Because the *in vitro* studies reveal only modest differences between the two enzymes, the activities of the two isoforms were examined in brain and leg muscle homogenates to determine if there are tissue-specific interactions that selectively alter the activities. The homogenates were centrifuged and a fixed amount of enzyme was added to either the supernatant or the pellet as described in Experimental Procedures. The background activity was measured at two different times, immediately after separation of the supernatant and resuspension of the pellet (T_0) and 45 min after separation and resuspension of the pellet (T_{45}). Because the background activity is much higher at T_0 than at T_{45} (Fig. 2), the enzyme was added to the appropriate cell fraction at T_{45}. Addition of nNOSμ, which is not found in the brain, to brain supernatant or pellet gives the same result as addition of nNOSα. The reciprocal experiment, addition of nNOSα to the leg muscle supernatant or pellet also yields similar results to those obtained by addition of nNOSμ (Fig. 2). There do not seem to be differential tissue effects on the catalytic rates of the two isoforms.

Time Dependence of nNOS Activity

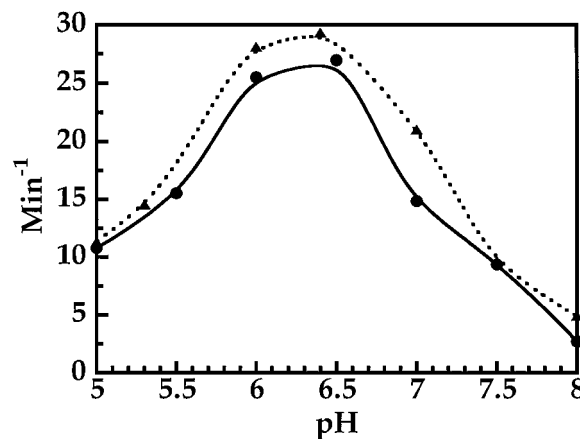The observation of a decrease in the background activity suggested that the endogenous NOS (and/or arginase) activ-

Fig. 1. The pH dependence of the L-Arg oxidizing activity of nNOSα (▲) and nNOSμ (●). The incubation details are given in Experimental Procedures.

ity in the cell homogenate is very time-dependent. To investigate the time dependence of the NOS activity, we incubated a fixed amount of both recombinant nNOSα and nNOSμ with the supernatant and pellet fractions from both the brain and leg muscle homogenates. As before, the enzyme was added to the mixture after a 45-min preincubation and the activity was subsequently determined at different time points (Fig. 3). The time-dependent changes in the activities are different for the two enzymes; nNOSμ is more stable than nNOSα in incubations with both the brain supernatant and resuspended pellet. Loss of 50% of the initial nNOSα activity is observed after 12 and 50 min of incubation with the resuspended pellet and supernatant, respectively, whereas loss of 50% of the nNOSμ activity requires 30 and 67 min, respectively. Incubation of nNOSα in the muscle homogenate and of nNOSμ in the brain homogenate do not give rise to behaviors for either of the enzymes that differ from those seen in their normal environments. In both the brain and leg muscle homogenates, the two isoforms have very short half-lives, that of nNOSα being significantly shorter than that of nNOSμ.

Digestion of NOS by Calpain

The time dependence of the activity of the nNOS isoforms in the cell homogenate is consistent with either thermal denaturation or proteolytic degradation. It has been reported that the catalytic activity of endogenous nNOS in rat brain supernatant decreases in a time-dependent manner that is not inhibited by the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and soybean trypsin inhibitor (Mittal and Jadhav, 1994). This suggested that the more common serine, aspartate, or thiol proteases might not be responsible for the observed loss of activity. We therefore examined the possible involvement of calpain, a Ca^{2+}-dependent protease (Molinari and Carafoli, 1997). Calpain is an attractive candidate for this activity because (a) Barnes and

Gomes (1995) reported that endothelial NOS has a potential calpain site, (b) Walker *et al.* (1996) recently obtained evidence in cell culture that iNOS may be a substrate for calpain, and (c) calpain can be both a soluble and membrane bound enzyme (Sato *et al.*, 1995; Suzuki *et al.*, 1995). Calpain is a Ca^{2+}-activated cysteine protease ubiquitously distributed in animal cells. A time-dependent study of the digestion of nNOSα (90 pmol) with the 80-kDa subunit of rabbit skeletal muscle calpain (2 pmol), monitored by Western blots using a monoclonal antibody against the reductase domain of nNOS, establishes that calpain readily digests nNOS (Fig. 4). After 10 min, nNOSα is completely digested to a 79.4-kDa fragment that is degraded, in turn, to a smaller peptide of ~45 kDa. The results suggest the presence of at least two calpain-sensitive sites in nNOS, the most sensitive of which results in complete digestion of nNOSα to a 79.4-kDa fragment within 10 min of incubation. The size of this first fragment suggests that the most critical calpain-sensitive site is near the middle of the protein in the vicinity of the CaM binding domain. Previous work on the digestion of nNOS by trypsin has shown that this protease also cleaves the enzyme in the CaM binding domain (Sheta *et al.*, 1994)

Aurintricarboxylic acid was recently reported by Posner *et al.* to be a calpain inhibitor (Posner *et al.*, 1995). To further test the role of calpain in the digestion of nNOSα and nNOSμ by the cell homogenate, we repeated the previous experiment
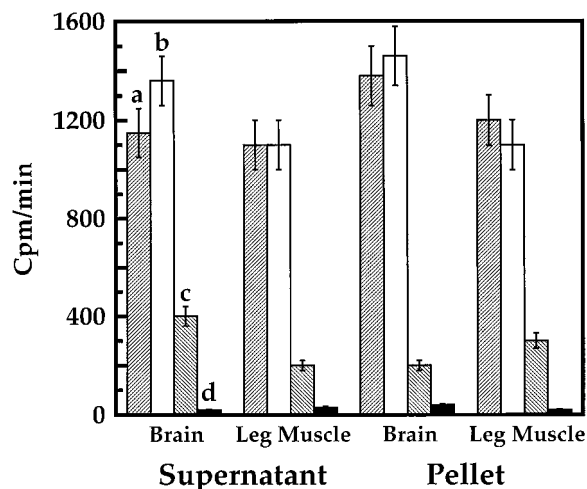
Fig. 2. Activities of nNOSα and nNOSμ measured in the presence of the supernatant or pellet of a rat brain or muscle homogenate. The four bars shown for each tissue and fraction represent the activities of nNOSμ in the presence of the indicated 45 min-preincubated cell fraction (▨, *a*), nNOSα in the presence of the indicated 45-min-preincubated cell fraction (☐, *b*), the background activity in the cell fraction immediately after preparation of the homogenate and fractionation into the supernatant and pellet by centrifugation (▧, *c*), and the background activity in the cell fraction after a 45-min preincubation (■, *d*). Purified recombinant nNOSα and nNOSμ were added to the 45 min-preincubated cell fractions and were immediately assayed for the production of citrulline from L-Arg.
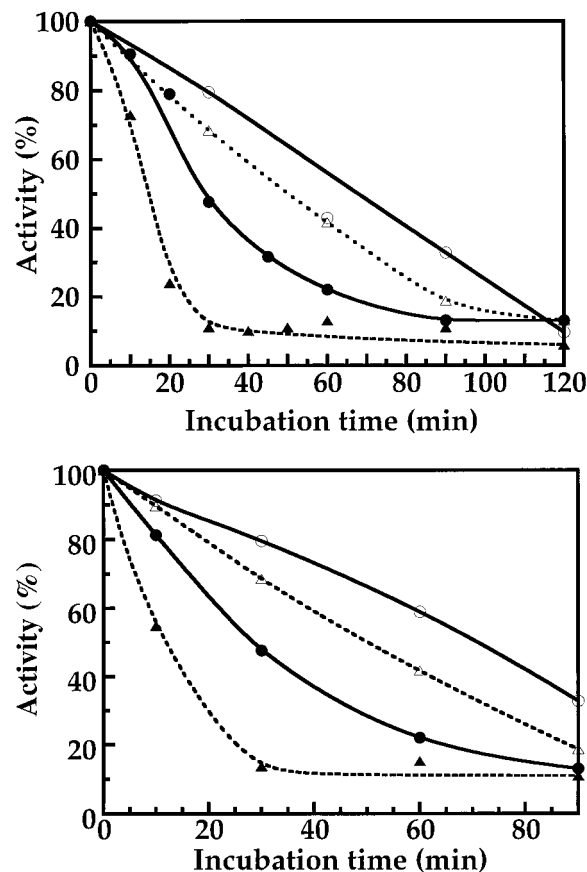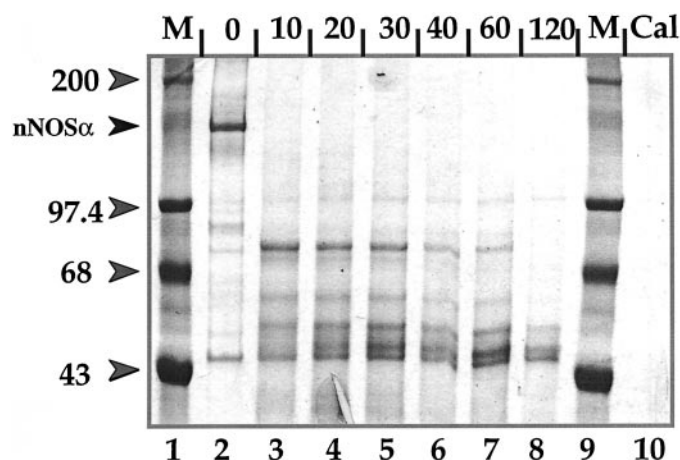

Fig. 3. Time-dependence of the L-Arg oxidizing activity of nNOSα and nNOSμ when incubated with the supernatant or pellet from a homogenate of (A) rat brain or (B) rat muscle. Incubation of nNOSα with brain supernatant (△) and brain pellet (▲), and incubation of nNOSμ with brain supernatant (○) and brain pellet (●). Full activity was 2100 cpm/min citrulline formation.

but in the presence of 500 μM aurintricarboxylic acid (Fig. 5). Aurintricarboxylic acid was added to the supernatant and the resuspended pellet after the 45 min preincubation but immediately before nNOSα or nNOSμ was added. Because the aurintricarboxylic acid might inhibit other proteases or otherwise alter the stability of the nNOS isoforms, we carried out similar experiments in the brain pellet with a very specific peptidic calpain inhibitor (Fig. 6). This peptidic inhibitor derives from the cellular calpain inhibitor calpastatin (Maki *et al.*, 1989). The results show that the half-lives of the two isoforms are greatly increased when calpain is inhibited. The half-life is > 4 hr in the brain pellet in the presence of the peptidic inhibitor and > 5 hr in the brain and leg muscle homogenates in the presence of aurintricarboxylic acid. Furthermore, these results indicate that loss of the enzymatic activity is not simply caused by thermal instability of the two proteins, as almost 80% of their activity is preserved after 3 hr under these incubation conditions. Calpain is thus clearly implicated as a key participant in the degradation of nNOSα and nNOSμ in the brain and in leg muscle, respectively.

Digestion of Endogenous nNOS by Calpain

As already mentioned, brain homogenates have an endogenous NO synthesizing activity when freshly prepared, but this activity decreases with time if the homogenates are incubated with ATP for a period of 30–45 min. To determine if this decrease in the activity of endogenous nNOS is caused by digestion by calpain, freshly prepared brain homogenates were incubated with and without the peptidic calpain inhibitor and the samples were analyzed by Western blotting for loss of immunodetectable nNOS. The results show that the nNOS detected by Western blotting of fresh homogenate is completely degraded after 2 hr incubation at 25° (Fig. 7), in accord with the earlier finding that the NO synthesizing activity is lost in a similar time period (Fig. 2). In contrast, only a modest loss of immunodetectable nNOS is found by Western blotting when the 2 hr incubation is carried out in the presence of the peptidic calpain inhibitor (Fig. 7). These results clearly show that endogenous calpain is the protease
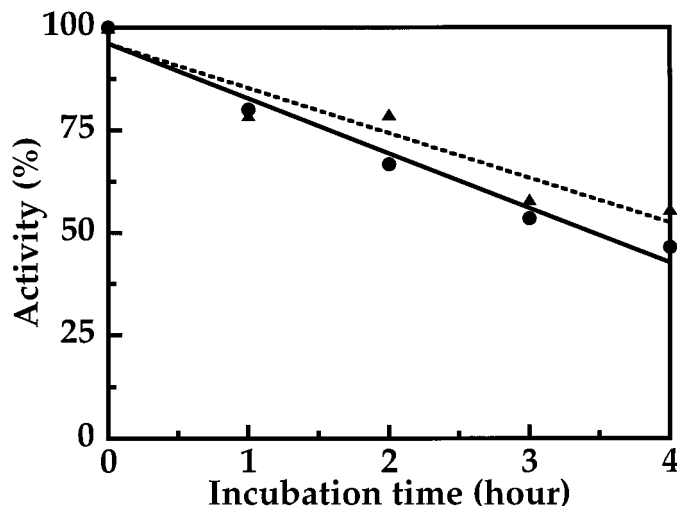
that is primarily responsible for the degradation of endogenous nNOS in brain tissue, although the fact that proteolytic peptides similar to those seen in Fig. 4 do not accumulate
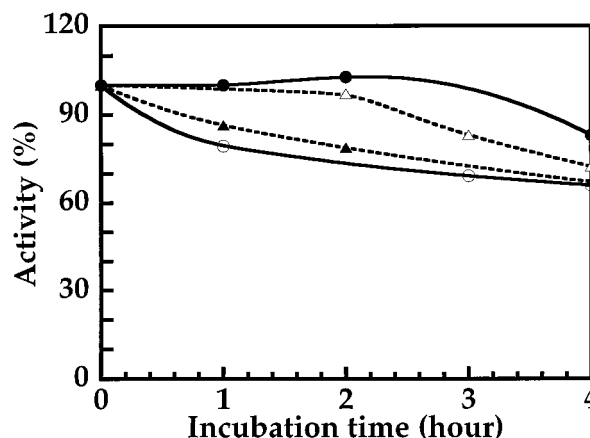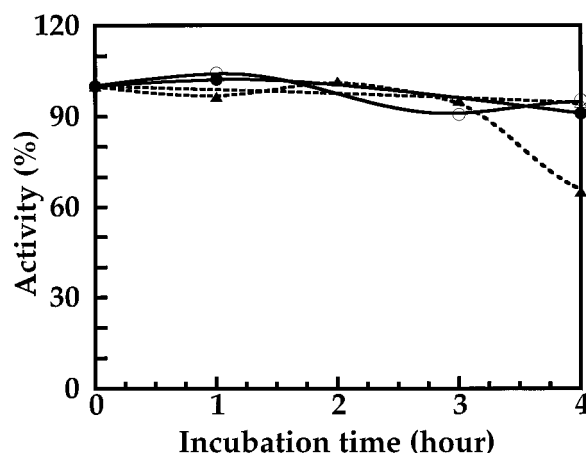
Fig. 5. Time-dependence of the L-Arg oxidizing activity of nNOSα and nNOSμ when incubated with the supernatant or pellet from a homogenate of (A) rat brain or (B) rat muscle in the presence of the calpain inhibitor aurintricarboxylic acid (Posner *et al.*, 1995); nNOSα with brain supernatant (△) or brain pellet (▲), and nNOSμ with brain supernatant (○) or brain pellet (●).

Fig. 4. Western blot of nNOSα and nNOSμ after digestion with calpain. The blot was developed with a monoclonal antibody against the reductase domain of nNOSα that is identical in sequence to that of nNOSμ. *Lanes 1 (M)* and *9 (M)*, marker proteins, the molecular masses of which are indicated on the *left* margin; *lanes 2–8,* nNOSα after incubation with calpain for the length of time (in min) indicated at *top*; *lane 10 (Cal)*, calpain.


Fig. 6. Time-dependence of L-Arg oxidation by nNOSα (- - - -) and nNOSμ (—|) when incubated with the pellet from a rat brain homogenate in the presence of a peptidic calpain-specific inhibitor (Maki *et al.*, 1989).

suggests that the peptides produced by calpain are rapidly digested by other proteases present in the brain tissue.

Discussion

Several isoforms have been identified that result from alternative splicing of the nNOS gene. Of these nNOS isoforms, nNOSα, nNOSβ, and nNOSγ are found in the brain (Huang *et al.*, 1993; Brenman *et al.*, 1996; Eliasson *et al.*, 1997), but nNOSμ is found in skeletal muscle (Magee *et al.*, 1996; Silvagno *et al.*, 1996). nNOSμ results from the insertion of a 34 amino acid peptide between the CaM and FMN domains of the nNOSα sequence. As shown here, this insertion has measurable but modest effects on some of the biochemical properties of the enzyme.

The k_{cat} and K_m values for the oxidation of L-Arg by the two isoforms, as suggested by the earlier study of the enzyme expressed in COS cells (Silvagno *et al.*, 1996), are essentially identical. The presence (or absence) of the peptide insert therefore does not influence the binding or oxidation of L-Arg by the two nNOS isoforms. However, although both nNOSα and nNOSμ consume NADPH at a high rate in the absence of substrate, the NADPH consumption by nNOSμ is half of that consumed by nNOSα even though the L-Arg oxidizing activities of the two isoforms are the same (Table 1). This implies that the 34-amino-acid insert modestly decreases uncoupled turnover, and consequently the formation of H_2O_2, in nNOSμ relative to nNOSα. These results agree with the finding that the rate of reduction of cytochrome *c* by nNOSμ is roughly half of that by nNOSα, a result that indicates that the flavoprotein domain of nNOSα gives up electrons more readily to both molecular oxygen and cytochrome *c* than the corresponding domain of NOSμ. The results also indicate that the communication between the reductase and heme domains in nNOSμ is not altered by the presence of the additional 34 amino acids. The lower electron donating activity of the reductase domain in nNOSμ does not affect the rate of L-Arg oxidation, presumably because the intrinsic electron donating capacity of both isoforms far exceeds the activity required for the relatively low rate of L-Arg oxidation (Table 1).
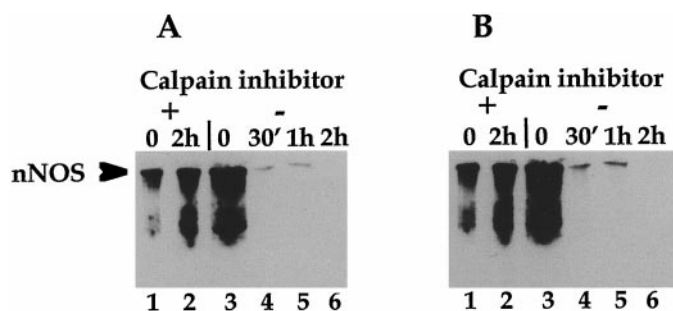
Fig. 7. Inhibition by the peptidic calpain inhibitor of the degradation of endogenous nNOS in brain homogenates. Fresh brain homogenates were incubated with and without the peptidic calpain inhibitor at 25° and aliquots were analyzed by Western blotting, as described in Experimental Preocedures. The sample in the presence of the inhibitor was diluted 1/50 before electrophoresis, whereas a 1/10 dilution was used in the absence of the inhibitor to increase the detectability of nNOS: *lane 1*, sample with inhibitor before incubation; *lane 2*, sample with inhibitor after 2-hr incubation; *lane 3*, sample without inhibitor before incubation; *lane 4*, sample without inhibitor after 30-min incubation; *lane 5*, sample without inhibitor after 1-hr incubation; *lane 6*, sample without inhibitor after 2-hr incubation. B is the same as A but was overexposed to improve the detection of weak bands. *Arrow*, nNOS band.

The NOS isoforms are flavin-containing hemoproteins that require the binding of CaM to trigger electron transfer from the reductase to the heme domains of the protein. The binding of CaM also greatly stimulates the reduction of cytochrome *c*, indicating that CaM binding not only brings about coupling of the reductase and heme domains but causes a change in the flavoprotein domain that enhances its ability to provide electrons. Location of the peptide insert between the CaM binding site and the FMN domain might be expected to alter either the binding of CaM or the electron transfer processes that are controlled by its binding. Our results indicate that the 34 amino acid insert has no influence on either of these parameters (Table 1). Thus, both isoforms have the same affinity for CaM, both require CaM binding for the oxidation of L-Arg, and in both enzymes CaM binding regulates the transfer of electrons to cytochrome *c* to approximately the same extent. The decreased activity of nNOSμ with respect to NADPH utilization and cytochrome *c* reduction thus stems from a direct effect of the insert on the reductase domain rather than from an effect mediated by the CaM binding site.

The pH-activity profiles of both nNOSα and nNOSμ exhibit bell shaped curves with optima at pH ~6.4. For both nNOS isoforms at least two groups control the pH dependence, one (or more) with a pK_a value of ~5.7 that needs to be deprotonated for maximum activity, and one (or more) with a pK_a value of ~7 that must be protonated for maximum activity. The similarity of the two pH profiles means that the 34-amino-acid insert has no influence on the pK_a values of the groups in question. The peptide insert therefore does not shift the pK_a of the nNOS protein to optimize it for function within a different subcellular environment.

These collective results show that insertion of 34 amino acids between the nNOS catalytic and reductase domains has only modest effects on the biochemical behavior of the enzyme and none on its ability to oxidize L-Arg. The two other known isoforms, nNOSβ and nNOSγ, retain only ~80% and ~5%, respectively, of the nNOSα L-Arg oxidizing activity (Brenman *et al.*, 1996). The amino-terminal domain of the enzyme deleted in these two isoforms seems to be important not only for interaction with other proteins but is also a determinant of the intrinsic NOS activity. In contrast, the modest catalytic differences between nNOSμ and nNOSα do not seem to be sufficiently important to provide a rationale for the differential expression of the two isoforms in brain and skeletal muscle.

The rapid differential degradation of nNOSα and nNOSμ, a key finding of the present study, is of greater significance than the small catalytic differences between the two isoforms. The 12 and 30 min half-lives of nNOSα and nNOSμ, respectively (Fig. 3), when incubated with the brain pellet, suggest that these nNOS isoforms may be among the more rapidly degraded proteins *in vivo*. The *in vivo* half-life of ornithine decarboxylase, a rapidly degraded protein, is ~11 min (Russell and Snyder, 1969). Digestion of nNOSα (and nNOSμ-not shown) by calpain (Fig. 4), and inhibition of activity loss by aurintricarboxylic acid (Fig. 5) and the calpain-specific inhibitory peptide (Fig. 6) in incubations of the nNOS isoforms with brain and muscle cellular fractions clearly implicate calpain as a major protease involved in their rapid degradation. The key role of calpain in the degradation of nNOS in native tissue is confirmed by the finding

that the proteolytic degradation of the endogenous nNOS in brain homogenate, as measured by Western blotting, is prevented by the peptidic calpain inhibitor (Fig. 7). Calpains are ubiquitous Ca^{2+}-dependent proteases that selectively cleave a wide variety of substrates. Furthermore, the earlier report that leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, and soybean trypsin inhibitor do not prevent the loss of nNOS activity in rat brain homogenates is consistent with a key role for calpain versus other proteolytic activities in triggering the inactivation of the nNOS isoforms (Mittal and Hadhav, 1994). The finding that calpain plays a key role in the degradation of nNOS is in accord with the report that calpain helps to limit the activity of iNOS in RAW 264.7 cells (Walker *et al.*, 1996).

Because ˙NO is a short-lived free radical, regulation of signaling occurs largely at the level of ˙NO synthesis. Both the nNOSα and nNOSμ isoforms are rapidly degraded by a Ca^{2+}-dependent protease; therefore, it is possible to envision a regulatory effect of protein degradation on ˙NO production. Activation of nNOS by Ca^{2+} would be followed by Ca^{2+}-dependent activation of calpain, which in turn would contribute to control of the level of ˙NO biosynthesis through nNOS catabolism. Proteolytic digestion of the enzyme, like its catalysis-dependent inactivation, provides a mechanism for preventing the elevated concentrations of ˙NO that are toxic to the cell.

The degradation of nNOS by calpain may play a significant role in Duchenne muscular dystrophy. It has been reported that nNOS is absent from the skeletal muscle sarcolemma of Duchenne muscular dystrophy patients (Brenman *et al.*, 1995; Chang *et al.*, 1996). nNOS is also deficient in the skeletal muscle of young mdx mice, an animal model for Duchenne muscular dystrophy (Chang *et al.*, 1996). Furthermore, calpain is overexpressed and activated in the skeletal muscle sarcolemma of deceased mdx mice (Spencer *et al.*, 1995; Spencer and Tidball, 1996). Our results suggest that elevated levels of active calpain may accelerate the degradation of nNOS and thus contribute to the deficiency of nNOS in the skeletal muscle sarcolemma of Duchenne muscular dystrophy patients and mdx mice.

In conclusion, the nNOSα and nNOSμ isoforms are biochemically closely related but are not identical. Of particular interest is the demonstration that nNOS is rapidly degraded by calpain, a Ca^{2+}-dependent protease. This degradation may be involved in regulation of the cellular concentration of ˙NO and may contribute to the deficiency of nNOS in some muscular disorders.

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