Interaction of Neuronal Nitric-oxide Synthase and Phosphofructokinase-M*

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Neurons that express neuronal nitric-oxide synthase (nNOS) are resistant to NO-induced neurotoxicity; however, the mechanism by which these neurons are protected is not clear. To identify proteins possibly involved in this process, we performed affinity chromatography with the nNOS PDZ domain, a N-terminal motif that mediates protein interactions. Using this method to fractionate soluble tissue extracts, we identified the muscle isoform of phosphofructokinase (PFK-M) as a protein that binds to nNOS both in brain and skeletal muscle. PFK-M interacts with the PDZ domain of nNOS, and nNOS-PFK-M binding can be competed by peptides that bind to the PDZ domain of nNOS. We found that nNOS is significantly associated with PFK-M in skeletal muscle because nNOS can be immunodepleted from cytosolic skeletal muscle extracts using an antibody directed against PFK-M. In brain, nNOS and PFK-M are both enriched in synaptosomes, and specifically, in the synaptic vesicle fraction, where they can interact. At the cellular level, PFK-M is enriched in neurons that express nNOS protein. As fructose-1,6-bisphosphate, the cellular level, PFK-M is enriched in neurons that express nNOS protein. As fructose-1,6-bisphosphate, the product of PFK activity, is neuroprotective, the interaction of nNOS and PFK may contribute to neuroprotection of nNOS positive cells.

Nitric oxide, derived from neuronal nitric-oxide synthase (nNOS), has important physiological functions in the nervous system (1, 2). In the peripheral nervous system, NO mediates certain actions of autonomic motor neurons on smooth muscle and thereby NO regulates intestinal peristalsis and penile erection. In the central nervous system, NO does not primarily act on smooth muscle but instead modulates synaptic transmission associated with N-methyl-D-aspartate (NMDA) type glutamate receptors. By regulating synaptic plasticity, NO can influence hippocampal long term potentiation (3) as well as aspects of learning (4) and memory (5).

Although small amounts of NO mediate physiological signaling, excess NO production can cause tissue injury (6). The primary stimulus for NO synthesis in central neurons is activation of NMDA receptors (2). Overactivity of NMDA receptors is implicated in numerous neurodegenerative processes including stroke, Huntington’s chorea, and amyotrophic lateral sclerosis (7). A role for NO in NMDA-mediated degeneration was first suggested by experiments showing neuroprotection by NO synthase antagonists (8). Definitive evidence that nNOS mediates brain injury derives from studies of nNOS knockout mice, which are strikingly resistant to excitotoxicity following focal cerebral ischemia (9).

NO can mediate neurotoxicity by inhibiting metabolic pathways and causing cellular energy depletion, which is a hallmark of ischemic injury-induced neuronal death. NO erodes energy stores by reacting with certain metabolic enzymes that contain heme-iron prosthetic groups, iron-sulfur clusters, or reactive thios (10). Through these reactions, NO can inhibit glycolysis by reacting with cis-aconitase and can attenuate oxidative phosphorylation by inhibiting mitochondrial iron-sulfur enzymes and by competing with oxygen at mitochondrial oxidase (11, 12). Interestingly, nNOS-containing neurons are themselves relatively resistant to excitotoxic injury, suggesting that nNOS neurons have specific mechanisms that protect them from energy depletion associated with NO toxicity (13).

Because NO mediates critical physiological functions but can also be toxic, nNOS activity must be tightly regulated. Selective regulation of calmodulin-dependent nNOS by specific calcium pools is mediated by the targeting of nNOS to subcellular sites (14). In brain, nNOS is targeted to the synaptic membranes through interactions with postsynaptic density-95 (PSD-95) and PSD-93 (15, 16). PSD-95 and PSD-93 are major proteins of the postsynaptic density and bind to nNOS via PDZ domains (PSD-95, discs-large, ZO-1) domains, consensus sequences of approximately 100 amino acids found in some cytoskeletal proteins and enzymes that mediate protein-protein interactions (17). The association of nNOS and PSD-95 is mediated by a direct PDZ-PDZ interaction (15). PDZ domains from nNOS and PSD-95 can also interact with specific sequences at the C termini of target proteins (18–21). PSD-95 and PSD-93 also synaptically cluster NMDA receptors, which are glutamate-activated calcium channels (22, 23). This macromolecular signaling complex is poised to regulate the synaptic activity of nNOS (14).

As nNOS occurs at both synaptic and nonsynaptic sites, we wondered whether other protein complexes might also bind the PDZ domain of nNOS. To address this, we performed affinity chromatography using a fusion protein column containing the PDZ domain of nNOS. Purification of brain lysates on this column yielded a protein band of approximately 80 kDa that was identified as an isoform of phosphofructokinase (PFK-M). Endogenous PFK-M is indeed associated with nNOS, and PFK-M and nNOS are both concentrated in a synaptic vesicle.
fraction from brain homogenates. At the cellular level, PFK-M is selectively enriched in cortical neurons that express nNOS. These data identify PFK as a major nNOS-interacting protein in brain cytosol. As PFK catalyzes the rate-limiting step in glycolysis and the product of this reaction, fructose-1,6-bisphosphate, is neuroprotective (24–26), PFK is a candidate enzyme for mediating neuroprotection of nNOS neurons.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were from Research Genetics (Birmingham, AL). Glutathione-Sepharose and pGEX4T-1 vector were from Amer sham Pharmacia Biotech. Affi-Gel 10 beads were from Bio-Rad. Horseradish peroxidase-coupled donkey anti-guinea pig antibody was from Sigma. ECL reagents were from Amersham Pharmacia Biotech. Mouse antibody raised against nNOS was from Transduction Laboratories (Lexington, KY). Guinea pig antibody raised against rabbit PFK-M was generously provided by Dr. Robert G. Kemp, University of Chicago. All other reagents were from Sigma.

**Expression and Purification of GST and GST-nNOS−195 Proteins**—A glutathione S-transferase fusion protein encoding the first 195 amino acids of nNOS was constructed by polymerase chain reaction and was expressed and purified from Escherichia coli as described previously (16). For protein affinity chromatography studies, GST fusion proteins were not eluted from the beads.

**Protein Affinity Chromatography**—Purified GST fusion proteins were dialyzed against PBS at 4 °C for 16 h. Five mg of protein was cross-linked to 1 ml of Affi-Gel 10 by incubating in PBS + 50 mM HEPES, pH 7.4, at 4 °C for 16 h on a shaking platform. Affinity chromatography was performed as described previously (27) with modifications. GST- and GST-nNOS-nNOS Affi-Gel beads were incubated in 0.1 ml Tris-HCl, pH 8.0, 350 mM NaCl on ice for 1 h. The affinity resin was then washed consecutively with Buffer C (50 mM HEPES, pH 7.6, 125 mM NaCl, 20% glycerol) containing 1 mM DTT, Buffer C containing 1 mM DTT and 2.5 M urea, Buffer C containing 1 mM DTT and 4 M urea, and Buffer D (20 mM HEPES, 125 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM EGTA). These washes precleared the columns of any unlinked GST fusion protein that might leach off the Affi-Gel beads during incubation with brain extract. Bovine serum albumin-Affi-Gel 10 beads were constructed in a similar manner and were used to clear the extract prior to its application to the GST- and nNOS-Affi-Gel 10 to reduce the background binding of nontpecific proteins.

Twenty-five adult rat brains were homogenized in ice-cold Buffer D, Triton X-100 (TX) was added to 1%, and proteins were extracted for 1 h at 4 °C. The homogenate was spun at 12,000 × g for 10 min, and the supernatant was precleared by incubating with 2 ml of bovine serum albumin-Affi-Gel 10 beads that were pre-washed with Buffer D at 4 °C for 2 h. The bovine serum albumin beads were removed, and 100 ml of extract was mixed with 0.5 ml of Affi-Gel 10 at 4 °C for 3 h. As controls, 10 ml of extract was incubated with 50 μl of GST-Affi-Gel 10, and 10 ml of Buffer D containing 1% TX was incubated with 50 μl of nNOS-Affi-Gel 10. All incubations were done in batch. Extract/beads were then loaded into the appropriate size columns and were washed three times with 20 ml TEE containing 0.2% TX. Bound proteins were eluted with 200 μl of 0.5% SDS and 100 mM NaCl, resolved on 10% polyacrylamide gels, and transferred to polyvinylidene difluoride membrane as described above. Blots were probed using a guinea pig antibody raised against rabbit PFK-M and visualized using a secondary antibody coupled to horseradish peroxidase and ECL reagents.

**Immunodepletion**—Skeletal muscle extract, prepared as described above, was precleared with 50 μl of protein A-Sepharose/ml at 4 °C for 1 h. The protein A-Sepharose was removed, and the extract was diluted 1:10 in TEE containing 1% TX and 1 mM PMSF. 5 μl of guinea pig anti-PFK-M or guinea pig preimmune serum was added to 1 ml of the diluted extract, and the extract was incubated at 4 °C for 1 h. Protein A-Sepharose (50 μl/ml extract) was added, and the extract was incubated at 4 °C for 1 h. The protein A-antibody complex was pelleted, and the resulting supernatant was separated on a 10% SDS-polyacrylamide gel. Protein was transferred to polyvinylidene difluoride as described above, and the blot was probed with a monoclonal antibody raised against nNOS.

**Synaptosomal Fractionation**—Synaptosomes were prepared as described by Li et al. (29) with modification. Four rat cortices were homogenized in 36 ml of homogenization buffer (320 mM sucrose, 4 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM PMSF) using 10 strokes at 900 rpm of a loose fitting glass-Teflon homogenizer (Kontes; size 22). The homogenate was centrifuged at 1000 × g for 10 min. The supernatant (S1) was collected and centrifuged at 12,000 × g for 15 min, and the pellet (P2) was resuspended in 24 ml of homogenization buffer and centrifuged at 13,000 × g for 15 min. The resulting pellet (P2′), representing a crude synaptosomal fraction, was lysed by osmotic shock and homogenized by three strokes of the glass-Teflon homogenizer at 2000 rpm, and the homogenate was spun at 33,000 × g for 20 min to yield supernatant (LS1) and pellet (LP1, heavy membranes). LS1 was spun at 251,000 × g for 2 h. The resulting supernatant (LS2) contained soluble proteins, and the pellet (LP2) contained synaptic vesicle proteins. Proteins were resolved on a 10% SDS-polyacrylamide gel, and Western blotting was performed as described above.

**Immunohistochemistry**—Adult rats were perfused with 4% paraformaldehyde, and brains were harvested, postfixed at 4 °C for 3 h, and cryoprotected in 20% sucrose overnight. Brains were sectioned sagittally at 20 μm with a cryostat, and sections were blocked for 1 h in

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**Fig. 1.** Affinity chromatography with the PDZ domain of nNOS. The PDZ domain of nNOS fused to GST was coupled to Affi-Gel 10 beads. Brain extract was incubated with nNOS-Affi-Gel 10 or GST-Affi-Gel 10. Buffer incubated with nNOS-Affi-Gel 10 served as a control. Proteins were eluted consecutively with 2.5 mM urea twice and 4 mM urea. Proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis, and the gel was visualized by silver staining. The arrow points to the 80-kDa doublet specifically eluted from the nNOS beads but not from the nNOS beads incubated in buffer or the GST control beads.
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Isolation of an 80-kDa Protein That Binds to the PDZ Domain of nNOS—To identify proteins that bind to the PDZ domain of nNOS in brain, we covalently coupled a nNOS PDZ domain GST fusion protein to Affi-Gel 10 (nNOS-Affi-Gel) and incubated this column with brain extract (nNOS-extract in Fig. 1). The column was washed with the buffer used to solubilize the brain extract, and proteins tightly bound to the column were eluted off sequentially with 2.5 and 4 M urea in buffer. Eluted proteins were compared with proteins that eluted from two control columns: first, a column of GST coupled to Affi-Gel 10 (GST-Affi-Gel) that was incubated with brain extract (GST-extract) to identify nonspecific binding proteins, and second, a column of the PDZ domain protein coupled to Affi-Gel 10 incubated with buffer (nNOS-buffer) to identify nNOS fusion protein that leached off of the column. As seen in Fig. 1, proteins of 45, 53, 60, and 65 kDa leached from the nNOS-Affi-Gel buffer control column (lanes 1 and 4). Additionally, a number of nonspecific proteins (31, 33, 35, 39, and 50 kDa) eluted from both the nNOS- and GST-Affi-Gel columns (lanes 2, 3, 5, and 6). These proteins either bind to GST or nonspecifically bind to Affi-Gel beads. The most abundant protein eluted specifically from the nNOS-column is a protein doublet at approximately 80 kDa (lanes 3 and 6). This doublet does not bind to a control GST column and is not a product of proteolysis of the linked fusion protein. This doublet as present in both the 2.5 and 4 M urea eluates (lanes 3 and 6). Fractions containing this doublet were combined, the more abundant upper protein was isolated, and a single peptide was sequenced by Edman degradation as described under “Experimental Procedures.” The sequence obtained (Fig. 2) showed high homology to the previously sequenced rat muscle phosphofructokinase (PFK-M, Fig. 2).

nNOS Binds PFK at Its Peptide-binding Site—It has been previously shown that the PDZ domain of nNOS preferentially binds to peptides ending with the sequence G/D/EIAV (19, 30) or EIAV (20). Interestingly, the final four amino acids of rat PFK-M are EAAV, a sequence similar to that of CAPON, a brain-specific protein isolated as a nNOS PDZ-binding partner (20), which ends with EIAV. To determine whether PFK-M binds to the peptide-binding site in the nNOS PDZ domain, we performed binding experiments with the PDZ domain of nNOS fused to GST. Fig. 3 (top panel) shows that PFK-M from both brain and skeletal muscle binds to the PDZ domain of nNOS. Furthermore, this binding can be competed with the peptide VSPDFGDAV, suggesting that PFK-M binding is to the peptide-binding site in nNOS-PDZ. Neither of the peptides, KLSS-IESDV, which binds to the second PDZ domain of PSD-95, and KLSSIEADA, blocks PFK-M binding to GST or GLSSIEADA interacts with PFK-M to the PDZ domain of nNOS (Fig. 3, middle panel). Experiments using the final nine amino acids of PFK-M fused to GST show that nNOS can bind to this C-terminal protein sequence (data not shown). In addition, VSPDFGDAV also competes the binding of CAPON to nNOS PDZ (Fig. 3, bottom panel). These results suggest that PFK-M binds to the nNOS-PDZ peptide-binding site, as does CAPON.

Immunodepletion of nNOS by PFK-M—To determine whether PFK-M and nNOS interact in vivo, we immunoprecipitated PFK-M from skeletal muscle extract. Skeletal muscle was used rather than brain because PFK-M is the only PFK isoform expressed in muscle (31). As shown in Fig. 4, immunoprecipitation of PFK-M depleted nNOS from the extract, whereas incubation of the extract with preimmune serum did not. These data suggest that PFK-M and nNOS interact in vivo and that a large fraction of nNOS in skeletal muscle cytosol is associated with PFK-M.

nNOS and PFK-M Are Both Concentrated in the Synaptic Vesicle Fraction—We also evaluated possible association of nNOS and PFK in brain. In order for these two proteins to interact, they must be co-localized. To determine whether nNOS and PFK-M are co-localized in brain, we performed subcellular fractionation (Fig. 5). In this preparation (29), cortical homogenate is centrifuged at low speed yielding a crude
nuclear fraction (P1). The supernatant (S1) is centrifuged providing a P2 pellet and S1 supernatant. The P2 pellet is washed to yield P2\textsuperscript{9}, a crude synaptosome fraction also containing mitochondria. After hypotonic lysis of P2\textsuperscript{9}, a plasma membrane fraction (LP1) containing pre- and postsynaptic membranes and the postsynaptic density was obtained. The soluble fraction was spun to obtain synaptic vesicles (LP2) and soluble cellular components (LS2). As seen in Fig. 5, nNOS is found in both soluble and particulate fractions. nNOS is enriched in synaptosomes (P2\textsuperscript{2}) and in the synaptic vesicle fraction (LP2), which is enriched in synaptophysin, a synaptic vesicle marker. PFK is also present in synaptosomes (P2\textsuperscript{2}) and in the synaptic vesicle fraction (LP2). In contrast, PSD-95 is concentrated in the synaptic membrane and PSD fraction (LP1).

![Fig. 4](image1.png)

**Fig. 4.** nNOS is immunodepleted from cytosolic skeletal muscle extract by immunoprecipitation of PFK-M. Guinea pig anti-PFK-M or guinea pig preimmune serum was added to cytosolic muscle extract, antibody complexes were collected with protein-A-Sepharose, and the immunoprecipitated extracts were separated on a 10% SDS-polyacrylamide gel. Western blotting for nNOS reveals an nNOS doublet of approximately 140–160 kDa; bands below the doublet are proteins that cross-react with the antibody. Note that the only protein that is immunodepleted by the antibody against PFK-M is the nNOS doublet.

![Fig. 5](image2.png)

**Fig. 5.** nNOS and PFK-M are enriched in the synaptic vesicle fraction of synaptosomes. Subcellular fractionation of brain was performed as described under “Experimental Procedures.” nNOS is concentrated in synaptosomes (P2\textsuperscript{2}) and in the synaptic vesicle fraction (LP2), which is enriched in synaptophysin, a synaptic vesicle marker. PFK is also present in synaptosomes (P2\textsuperscript{2}) and in the synaptic vesicle fraction (LP2). In contrast, PSD-95 is concentrated in the synaptic membrane and PSD fraction (LP1).

**DISCUSSION**

The primary finding of this study is that PFK-M is associated with nNOS in brain and skeletal muscle. PFK-M binds to the PDZ domain of nNOS, and this binding can be competed with a peptide, VSPDFGDAV, that also specifically binds to the PDZ domain of nNOS. Like the binding of PFK-M, the binding of CAPON (20) to the PDZ domain of nNOS can also be competed by the VSPDFGDAV peptide, suggesting that the two proteins bind to the same site in the nNOS PDZ domain. We have also shown that PFK-M and nNOS associate in vivo because immunoprecipitation of PFK-M from cytosolic muscle extract depletes nNOS. Furthermore, many cortical neurons that are enriched in nNOS are also enriched in PFK-M. This was seen in 15 of 15 cortical neurons and 12 of 12 striatal interneurons examined.

**PFK-M Is Enriched in Neurons That Express nNOS**—nNOS is expressed in a discrete subpopulation of interneurons in the corpus striatum and cerebral cortex. To determine whether PFK-M is present in these neurons, we immunostained for both nNOS and PFK-M in adult rat brain sections. As seen in Fig. 6, cortical interneurons that express nNOS are enriched in PFK-M. This was seen in 15 of 15 cortical neurons and 12 of 12 striatal interneurons examined.

**FIG. 6.** PFK-M is enriched in cortical neurons that express nNOS. 20-μm sections from adult rat brain were stained for nNOS (top) and PFK-M (middle), and nuclei were visualized by staining with 4’,6’-diamidino-2-phenylindole (DAPI, bottom). Sections were examined by fluorescence microscopy using a 20× objective. Arrows point to cells that are enriched in both nNOS and PFK-M. Control experiments lacking anti-PFK-M and anti-nNOS yielded no staining. The scale bar is equal to 10 μm.
brain (32) and is co-expressed with the two other isoforms of PFK in the brain where these three isoforms form heterotetramers (31, 33, 34). PFK-M is the only isoform that ends in the consensus for nNOS PDZ binding (20), and hence is likely to be the only isoform that can bind to nNOS directly. We have shown that PFK-M binds to nNOS at a peptide-binding site in the PDZ domain of nNOS (Fig. 3) as PFK-M can be competed off with a peptide that binds to this peptide-binding site and that competes with the binding of CAPON.

Because PFK-M is the only isoform found in muscle, it serves as a good tissue in which to study the association of PFK-M and nNOS in vivo. Our data demonstrate that PFK-M binds to nNOS from skeletal muscle extracts and that a large fraction of nNOS in muscle cytosol associates with PFK (Figs. 3 and 4). This interaction may be functionally important because NO can regulate energy metabolism in normal muscle. In rodent myofibers, nNOS is specifically enriched at neuromuscular endplates (16) and in fast twitch fibers, which are known to be glycolytic (35). Exercise-stimulated glucose transport (36) and cytokine-modulated glucose transport (37) are dependent on NO. It is possible that in glycolytic fast twitch fibers, nNOS regulates both glucose transport and PFK function.

We wondered whether the nNOS-PFK-M interaction might directly regulate enzyme activities. However, we found that PFK activity of skeletal muscle from nNOS−/− mice is not different from that of wild type mice, nor does addition of a glutathione S-transferase protein encoding the PDZ domain of nNOS to skeletal muscle extracts change PFK activity. PFK activity does not affect nNOS activity because adding brain lysate containing PFK activators has no effect on nNOS activity. With this in mind, we suspect that PFK-M interactions help determine the disposition of cytosolic nNOS rather than directly regulate enzyme activity.

In brain, nNOS is targeted to the plasma membrane and postysnaptic density by PSD-95 and PSD-93 proteins via PDZ-PDZ interactions (15, 16), whereas PFK-M is a cytosolic protein and may target nNOS to the cytosol. In fact, a significant fraction nNOS in brain is soluble (38). In addition, the catalytic subunit of protein phosphatase-1 is targeted to PFK-M via a protein-protein interaction (39), and thus, the binding of PFK-M to the PDZ domain of nNOS may target nNOS to a cytosolic complex of enzymes.

By subcellular fractionation of brain homogenates, we find that both nNOS and PFK-M are enriched in synaptosomes and, more specifically, in a synaptic vesicle fraction (Fig. 5). These data are consistent with electron microscopic data that nNOS is present at nerve terminals in the cerebral cortex (40) and the nucleus accumbens (41) of the rat where NO can serve to preserve ATP levels during hypoxia in neonatal brain slices (25). In aging rats, ischemic-induced memory dysfunction can be reversed by dimethyl sulfide-FBP, which serves to scavenge free radicals (26). Thus, nNOS-expressing neurons may express high levels of FBP because they are enriched in PFK-M. Furthermore, FBP activates PFK via a feed-forward mechanism, resulting in amplification of FBP production. Thus, nNOS positive neurons may be protected from NO-induced neurotoxicity via a FBP-dependent mechanism.

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