

PIN/LC8 is associated with cytosolic but not membrane-bound nNOS in the nitrergic varicosities of mice gut: implications for nitrergic neurotransmission

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RUNNING HEAD: PIN/LC8 binds with cytosolic, but not membrane-bound nNOS.

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ABSTRACT

This investigation demonstrates the presence and binding of the protein LC8 (described as “protein inhibitor of nNOS” or PIN in some reports) to different components of nNOS in nitrergic varicosities of mice gut. Whole varicosity extracts showed three (320, 250 and 155kDa) nNOS bands with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody and a 10kDa band with anti-LC8 antibody. The LC8-immunoprecipitate (IP) showed three nNOS bands, suggesting that LC8 was bound with all three forms of nNOS but dissociated from them during SDS-PAGE. Studies using LC8-immunoprecipitate and supernatant and probed with anti-CaM showed that LC8 was not associated with CaM-bound 320kDa nNOS, but was present in CaM-lacking fraction. Probing these fractions with anti-serine847-P-nNOS showed that 320kDa serine847-phosphorylated-nNOS consisted of LC8-bound and LC8-lacking components. Subsequent studies with varicosity membrane and cytosolic fractions separately showed that membrane contained CaM-bound and CaM-lacking, serine847-phosphorylated 320kDa nNOS; both these fractions lacked LC8. On the other hand, the cytosolic fraction contained CaM-lacking, serine847-phosphorylated 320kDa, 250kDa and 155kDa nNOS bands that were all associated with LC8. These studies, along with *in vitro* nitric oxide assays, show that in gut nitrergic nerve varicosities: 1) all cytosolic serine847 phosphorylated nNOS was catalytically inactive and bound with LC8. 2) Membrane-associated nNOS consisted of catalytically active, CaM-bound and catalytically inactive, CaM-lacking, serine847-phosphorylated nNOS α dimers, both of which lacked LC8. These results suggest that LC8 may dissociate from the 320kDa nNOS α dimer upon binding to membrane, thus supporting the view that LC8 may

transport nNOS α dimer to the varicosity membrane for participation in nitreergic neurotransmission.

KEY WORDS protein inhibitor of nNOS (PIN), dynein light chain (DLC8 or LC8), nNOS, membrane-bound nNOS, cytosolic nNOS, nitreergic neurotransmission

INTRODUCTION

“Protein inhibitor of nNOS” (PIN), an 89 amino acid, 10kDa protein, was so named because it was thought to negatively regulate nNOS (neuronal nitric oxide synthase) activity.(8) PIN was shown to selectively bind and inhibit nNOS and not other isoforms of NOS such as eNOS or iNOS.(8) PIN was suggested to inhibit nNOS activity by promoting catalytically inactive nNOS monomer formation from catalytically active nNOS dimers. PIN was subsequently identified as a member of the light chains (LC8) of two motor proteins, namely, dynein and myosin Va.(3, 9). Therefore, the terms PIN and LC8 are used interchangeably. LC8 may play an important role in the intracellular transport of various subcellular proteins including nNOS and their targeting to their sites of action.

LC8 has ubiquitous cellular distribution and binds with multiple natural protein partners.(11, 13) It has been proposed that LC8 may form a major hub for protein-protein interaction (1) In biological systems, LC8 exists as a dimer that has two binding grooves.(12, 22). Each of these grooves may bind a 17-amino acid protein stretch (²²⁸Met to ²⁴⁴His of nNOS at the N terminal), leading to formation of a homodimer of nNOS (4). On the other hand, one groove of LC8 dimer may bind with the intermediate chain of dynein or myosin and the other one to nNOS and other cargo proteins being transported.(3)

The role of LC8 in the regulation of nitrergic neurotransmission is not well understood. On one hand, it may inhibit nitrergic neurotransmission by negative regulation of nNOS

activity.(8) On the other, it may facilitate nitrenergic neurotransmission by linking nNOS to the motor proteins and transporting it to the nerve terminals and the varicosity membrane.(6, 13)

Although PIN was initially thought to be an inhibitor of nNOS, its effect on nNOS activity is controversial. Jaffrey and Snyder (1996)(8) had initially shown that addition of PIN or GST-PIN to cell extract with over-expressed nNOS resulted in decreased nNOS activity. Hemmens et al. (1998)(6) have shown that the inhibitory effect of GST-PIN on purified active nNOS was not selective as other types of NOS were also inhibited. Moreover, Rodriguez-Crespo (1998)(17) found no effect of PIN on nNOS activity.

Effect of PIN on dimer:monomer state of nNOS is also controversial. Jaffrey and Snyder (1996)(8) had suggested that PIN may act by destabilizing nNOS dimers to catalytically inactive monomers. This conclusion was based on their findings that cell extract from nNOS-overexpressed cells had both 320kDa dimers and 155kDa monomers, but when GST-PIN was added, only the 155kDa monomers were seen. nNOS α dimer-monomer equilibrium has been proposed as an important mechanism of regulation of nNOS activity.(5) However, the role of PIN in destabilizing nNOS dimer to promote its monomerization is not supported by other studies.(14, 17, 23)

Intracellular nNOS *in vivo* may exist in different forms based on alternative splicing of its mRNA, dimer vs. monomer state, binding with other proteins such as CaM and its phosphorylation at serine847.(5, 7, 16, 18) The different forms of nNOS may differ in

their subcellular localization and their catalytic activity to produce nitric oxide.

Information on the association of LC8 with different forms nNOS may help understand the role of LC8 in nitrergic neurotransmission in the nerve terminals of gut. The present studies were performed to: 1) determine if LC8 was present in the nitrergic varicosities of mice gut; 2) identify the forms of nNOS that were associated with LC8; and 3) determine the nature of membrane-bound and cytosolic nNOS and their association with LC8. In addition, *in vitro* nitric oxide (NO) production assays were used to corroborate the functional status of LC8-bound and LC8-lacking fractions of nNOS.

MATERIALS AND METHODS

The experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of VA Boston Healthcare System.

Antibodies and Chemicals. The antibodies used in the present study are as follows: anti-LC8 (anti-PIN FL-89), 1:75 (Santa Cruz), anti-C-terminal-nNOS₁₄₂₂₋₁₄₃₃, 1:50 (Alexis), anti-serine847-P-nNOS, 1:50 (Santa Cruz), anti-CaM, 1:100 (Upstate), anti-synaptophysin, 1:25-1:100 (Santa Cruz), anti-LDH, 1:25 (Santa Cruz) All species-appropriate secondary antibodies were obtained from Santa Cruz and were used at a three-five fold dilution to the primary antibody. Reagents for immunoblot and different chemicals used were from BioRad, Sigma and Cayman Chemicals. Protein A/G was obtained from Santa Cruz.

Tissue Dissection. Adult male C57BL/6j mice (25-30 gm each) (Jackson Laboratories) were euthanized by carbon dioxide (CO₂) inhalation in an airtight chamber. Gastrointestinal tissues from six mice were pooled for each experiment. The entire gastrointestinal tract from the stomach to colon was dissected quickly and the gut lumen was cleaned in ice-cold homogenization buffer (0.3M sucrose with 0.1M sodium phosphate). Small pieces of intestine were placed in a plastic tube in 10 volumes of the homogenization buffer and were pulverized into a homogenous extract with a Brinkmann homogenizer and the tube cooled on ice before further processing. The homogenization buffers contained protease and phosphatase inhibitors (The phosphatase inhibitor was used at a concentration of 1 ml /100 ml of homogenization buffer. The protease inhibitor was used at a concentration of 1 ml /20 gm wet tissue. The complete gastrointestinal tract from each animal had an average mass of 2.8 gm, n=6 mice). The protease inhibitor (P8340, Sigma) contained AEBSF, aprotinin, bestatin, E-64, leupeptin hemisulfate and pepstatin. The phosphatase inhibitor contained cantharidin and microcystin LR (P2850, Sigma) that specifically inhibited serine phosphatase PP2A.

Subcellular fractionation. Samples were centrifuged at 1000 g for 10 min at 4° C to remove undissociated tissue (pellet P1) that was washed once in buffer and the pellet discarded and the combined supernatants was further centrifuged at 4000 g. The pellet P2 obtained after spinning at 4000 g represented the nuclear fraction and the supernatant was the cytoplasmic fraction. This supernatant was subjected to ultra-centrifugation at 25000 rpm at 4⁰C for 30 minutes in an Optima TLX cold ultracentrifuge. The Pellet P3 was the varicosity fraction, while the supernatant represented the microsomal fraction. Pellet P3

was re-suspended in 400 μ l of Krebs buffer (111mM NaCl, 26.2mM NaHCO₃, 1.2mM NaH₂PO₄, 4.7mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 11mM glucose) and subjected to further purification. The P3 extract was layered on a 0.8/1.2M sucrose gradient and subjected to sucrose gradient ultra-centrifugation at 58,000 rpm for 1hour at 4⁰C. Intact varicosities that formed a cloudy or ring-like structure at the interface of the two differing sucrose concentrations were carefully collected with a 200 μ l pipette tip, diluted in Krebs buffer and centrifuged at 12,000 rpm for 5 minutes at 4⁰C to pellet down varicosities. Varicosities were stored at -80⁰C until further experiments.

Separation of membrane and cytosolic fractions of synaptosomes. The purified varicosity lysate obtained after sucrose-gradient centrifugation was incubated in a two-volume solution of 0.5mM sodium phosphate (pH=8.1) and 0.1mM magnesium sulfate for 6 h on ice. This protocol was adapted from previously standardized methodology of preparation of unfolded red blood cell membrane by incubation in chilled alkaline buffer of very low ionic strength.(21) The divalent magnesium ions facilitated non-sealing of membranes. After incubation, the lysate was subjected to high-velocity differential centrifugation, as described earlier for membrane protein preparation,(20) using a speed of 70,000 rpm for 1 h at 4^o C. The supernatant represented the cytosolic fraction whereas the yellowish-white pellet represented only membranes of the varicosities.

Preparation for Western Blots. The extracts were processed at low temperature (4⁰ C) or heat treated at 37⁰ C for 10 minutes. For the low temperature processing, 60-80 μ g of protein in standard Laemmli buffer at 4⁰ C was used for SDS-PAGE. The low

temperature process was used to identify nNOS dimers and monomers in the native state as low temperature is known to prevent monomerization of nNOS dimers.(13) Heat treated samples were processed as follows: protein was treated with Laemmli buffer for 10 minutes at 37 °C and immediately subjected to electrophoresis. 35 µl of protein samples were loaded into each lane during electrophoresis.

SDS-PAGE. Electrophoresis was carried out using Biorad mini-protean II system gel casting system. Experiments were carried using 7.5% glycine gels. For detection of proteins with MW<20 (PIN and calmodulin), 10-20% tricine peptide gels were used, as tricine gels have been reported to provide enhanced resolution of very low molecular weight proteins.(19) For tricine gel experiments, the sample buffer used was 10% Tris/tricine/SDS and SDS/glycine buffer was used during electrophoresis. Electrophoresis was started at 60 V and stepped up to 90 V after the samples crossed the stacking gel. The runtime was determined based on the migration pattern of the molecular weight markers. Extensive pilot experiments were performed for standardization of the type of gel, the run-time, the concentrations of the primary and secondary antibodies and the incubation times.

Total protein concentrations were measured using Bradford method at 595λ optical density. For almost every experiment, 60µg of protein was loaded for low temperature SDS/PAGE. The samples were subjected to SDS/PAGE for a variable period of time (between 2 and 5 hrs, depending on the migration of the MW marker) at 90 V in cold-room at 4°C. For all electrophoresis, Precision Plus (BioRad) molecular weight marker

was used to identify migration patterns. All experiments were performed with appropriate positive controls and loading controls were evaluated for homogeneity of results. Additionally, for each experiment, three different sets of varicosity extracts were always used to compare results (each extract from a set of 6 mice) and electrophoresis was repeated twice to confirm the findings.

After all electrophoresis, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad) overnight at 30 V at 4⁰C in a glycine buffer medium and the efficiency of immunoblotting was checked using Ponceau staining. Blots were washed with Tris-buffered saline (BioRad) with Tween (TBST) for 10 minutes and blocked for 1 h in 5% bovine serum albumin (BSA) at room temperature. The primary antibody was added in the blocking solution at differing dilutions and the blot was probed with this primary antibody on a rocking platform overnight at 4° C. The blot was subsequently washed with washing buffer for a few times (the steps were standardized according to the individual experiments) and an appropriate secondary antibody conjugated to chemiluminescent horse radish peroxidase (HRP) was added and incubated for an hour on a rocking platform at room temperature. After washing the blot with TBST thrice for ~20 min each/wash, immunolabeled blots were developed using an enhanced chemiluminescent developer ECL reagent (GE Healthcare) or a Visualizer spray (Millipore). Blots were exposed to a chemiluminescent detection film in the dark for variable time periods and processed in an X-Ray developer machine (Kodak X-OMAT 2000A).

Immunoprecipitation studies. Solubilized varicosity extracts, the separated membranes and cytosolic fractions of varicosities were diluted to a starting protein concentration of 1mg/ml using a buffer that resembled composition of the standard RIPA buffer. This buffer contained protease and phosphatase inhibitors and 50mM Tris-HCl pH 7.5 (5ml), 150mM NaCl (3.75ml), 1% Nonidet P40 and 0.5% sodium deoxycholate (2.5ml). 50µl of a homogenous suspension of Protein G-agarose (A/G) was added to 1 ml varicosity extract and incubated overnight at 40% speed at 2-8⁰ C on a rotating rocker (Glas-Col, Terre Haute). Varicosity extracts with beads were centrifuged at 12,000g for 20 seconds in a microfuge. After the supernatant was transferred to fresh tubes, 10 µg of the specific antibody was added and gently rocked overnight at 2-8⁰C. After the immunoprecipitates were collected by centrifugation at 12,000g for 20seconds in a microfuge, the supernatant was carefully aspirated and stored for further experiments. The beads were washed thrice with 1 ml of lysis buffer and spun at 12000 g for 2 min each time and 2X loading buffer (the Laemmli buffer) was added to this protein A/G beads on ice to isolate protein from the beads. Immunoprecipitated protein was subjected to different types of electrophoresis and probed with different antibodies.

Quantification of blots. After developing, certain blots were scanned with unaltered luminosity so that the optical density remained unaffected. The peak intensity of a user-identified band was evaluated using the NIH program ImageJ. The band intensity was expressed as arbitrary units and mean expression was obtained by averaging intensity values of 3 different lanes for each experiment, each lane representing gut varicosity

extract from 6 mice. Expression values of these signals were represented as means \pm SEM. Comparison of means was performed using t statistics using MS Excel.

Functional studies of in vitro Nitric Oxide Production. NO production by LC8-bound and LC8-lacking fractions of nNOS (using immunoprecipitated gut nerve varicosity extracts with anti-LC8 antibody, and the supernatants) was measured by using fluorescence of DAF-2-DA (diaminofluorescein diacetate) at 495/515 nm in a spectrofluorometer (Biomate, Thermo Fischer Scientific). 100 μ l of immunoprecipitated protein samples was mixed with 100 μ l of assay buffer. The assay buffer consisted of 55mM N-2 hydroxyethylpiperazine-N-2-ethane-sulfonic acid (Hepes), 20mM Tris-HCl, pH 7.4, 2mM L-Arginine, 0.8 mM dithiothreitol (DTT), 0.8 μ M NADPH, 1mM MgCl₂, 1mM CaCl₂, 0.5 μ M calmodulin, 0.8 μ M riboflavin monophosphate (FMN), 6 μ M (6R)-5,6,7,8-tetrahydrobiopterin (BH₄), 0.8 μ M flavin-adenine dinucleotide (FAD), and protease inhibitors. DAF-2 (10 μ M) was added to this reaction mixture at the time of incubation. The assays were performed in triplicates under different experimental conditions using the native protein, preincubating with the nNOS inhibitor L-NAME and by omitting L-arginine in the buffer. The fluorescence intensity produced by known concentrations of the nitric oxide donor Nor-3 was used for plotting a standard graph and nitric oxide production of the unknown biological samples were calculated as molar equivalents of Nor-3 and expressed per mg of protein samples.

RESULTS

A. Studies in whole varicosity extracts

1. *LC8 is present in nitrergic nerve terminals.* Varicosity extracts processed at low temperature showed 3 bands of 320kDa, 250kDa and 155kDa reactive with nNOS₁₄₂₂₋₁₄₃₃. The 320kDa and 250kDa bands were dimers as evidenced by the fact that they were converted to 155kDa and 135kDa monomers on heat treatment of the extract at 37° C in SDS containing Laemmli solution (Figure 1A). It is known that SDS at warm temperature monomerizes nNOS dimer. Western blots of varicosity extracts probed with anti-nNOS₁₄₂₂₋₁₄₃₃ (C-terminal) antibody identify all isoforms of nNOS because the C-terminal regions of all isoforms of nNOS are identical. Western blots of the varicosity extracts using anti-LC8 antibody showed no LC8 staining with bands in the region of the nNOS. However, a 10kDa band was visualized with this anti-LC8 antibody (Figure 1B). These observations suggest that LC8 was present in the varicosities but was dissociated from its other protein partners during SDS PAGE. The extracts used for the above experiments showed a 42kDa band reacting with anti-synaptophysin antibody, indicating that the samples used were obtained from nerve varicosities (Figure 1C).

2. *LC8 is associated with nNOS in the varicosities.* In order to study whether LC8 was associated with nNOS in the varicosities, immunoprecipitates of varicosities with anti-LC8 antibody were probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody. SDS-PAGE of LC8- IPs at low temperature showed 3 bands at 320, 250 and 155kDa respectively (Figure 2). These results indicated that LC8 was associated with both dimers and monomers of nNOS.

3. *LC8 is not associated with CaM (calmodulin)-bound nNOS.* It has been shown that a fraction of nNOS in the varicosities exists as CaM-associated active nNOS.(14) In order to identify whether LC8 was associated with CaM-bound fractions of nNOS, varicosity extracts were immunoprecipitated with CaM antibody. The CaM-immunoprecipitate was probed with anti-nNOS₁₄₂₂₋₁₄₃₃ and anti- LC8 antibodies. The CaM immunoprecipitates showed a 320kDa nNOS reactive band (Figure 3A) but no LC8 reactive band (Figure 3B).

In reverse experiments, varicosity extracts were immunoprecipitated with LC8 antibody. The LC8-immunoprecipitate did not react with the anti-CaM antibody (Figure 3C). These observations show that LC8 was not associated with CaM-bound nNOS dimers.

The LC8-lacking fraction obtained after immunoprecipitation with LC8 antibody showed a 320kDa nNOS band during cold SDS-PAGE when probed with an anti-nNOS₁₄₂₂₋₁₄₃₃ antibody and a 155kDa band when the samples were warmed at 37° C prior to loading (Figure 3D). This suggested that a component of the 320 kDa nNOS α may not be associated with LC8. It also reacted with anti-CaM antibody and showed a 17kDa band (Figure 3E). These observations suggest that LC8 does not associate with CaM-bound nNOS in the nerve terminals.

4. *LC8 is associated with CaM-lacking nNOS.* In order to identify whether LC8 associated with CaM-lacking fractions of nNOS, the CaM-free supernatant was examined after immunoprecipitation of varicosity extracts with CaM. The CaM-lacking fraction

was probed with anti-nNOS and anti- LC8 antibodies; it showed 3 bands of 320kDa, 250kDa and 155kDa nNOS bands during low temperature SDS-PAGE (Figure 4A). Warm SDS-PAGE of the CaM-lacking fraction showed a 10kDa LC8 reactive band (Figure 4B). These observations suggested that PIN was associated with CaM-lacking nNOS bands.

In reverse experiments, varicosity extracts were immunoprecipitated with LC8 antibody and probed with anti-CaM and anti-nNOS₁₄₂₂₋₁₄₃₃ antibodies (Figures 3C and 2 respectively). These results showed that LC8-bound nNOS fractions included 320 kDa, 250 kDa and 155 kDa bands and none of these bands reacted with anti-CaM antibody, further showing that LC8-bound nNOS was not CaM-bound.

5. LC8 is associated with a component of serine847 phosphorylated nNOS. It has been reported that CaM-lacking nNOS may be serine847phosphorylated.(14) In order to test whether LC8-bound nNOS was serine847 phosphorylated, varicosity extracts were immunoprecipitated with LC8 antibody. The immunoprecipitate and the supernatants were probed with anti-serine847-P-nNOS antibody. The LC8-immunoprecipitate showed 3 phospho-nNOS bands (Figure 5A). Interestingly, the supernatant also showed a 320kDa phospho-nNOS (Figure 5B).

In reverse experiments, the varicosity extract was immunoprecipitated with serine847-P-nNOS antibody and probed with anti- LC8 antibody. Anti- LC8 antibody identified a 10kDa band (Figure 5C).

These observations suggested that in the varicosity there were two fractions of serine847-phosphorylated 320kDa nNOS: one of these fractions was associated with LC8 and the second component was not associated with LC8.

B. Studies in membrane bound and cytosolic fractions

The LC8-associated and LC8-unassociated components of 320 kDa nNOS may be related to the association of the nNOS components with the varicosity membrane. Therefore, we performed studies on membrane bound and cytosolic nNOS and their association with LC8.

Confirmation of separation of membrane-bound and cytosolic fractions. The purity of plasma membrane fractions was assessed by performing Western blotting against lactate dehydrogenase (a cytosolic marker) and purity of fractions was over 95%. Purity of cytosolic fractions was assessed by performing Western blotting against synaptophysin (a plasma membrane marker) and purity of fractions was 97% (Figures 6A & 7A).

6. Membrane bound nNOS isoforms. The nature of the membrane-bound and cytosolic nNOS isoforms was first investigated. The membrane fraction of the varicosities showed only a 320kDa band when probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody (Figure 6A). This band represented the dimer of nNOS α . Subsequently, it was examined whether this 320kDa band was associated with calmodulin or not. The membrane extracts were immunoprecipitated with anti-CaM antibody and both the immunoprecipitate and the supernatant were probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody. Both the anti-CaM

immunoprecipitate and the CaM-lacking supernatant of membrane extract showed 320kDa nNOS bands (Figure 6B). The CaM-bound and CaM-lacking fractions were also probed for reactivity with anti-serine847-phosphorylated nNOS. The CaM-bound nNOS 320kDa band did not react with anti-phospho-nNOS antibody, whereas the CaM-lacking fraction showed 320kDa phospho-nNOS band (Figure 6B). These observations suggest that the membrane of varicosities contains 2 different types of nNOS α dimer: a 320kDa band that is CaM-bound and another 320kDa band that lacks CaM and is phosphorylated at serine847.

7. LC8 is not associated with either CaM-bound or CaM-lacking nNOS fractions bound to varicosity membrane. To assess whether membrane-bound nNOS was associated with LC8, solubilized membrane proteins were immunoprecipitated with anti-LC8 antibody. Subsequent probing with an anti-nNOS₁₄₂₂₋₁₄₃₃ antibody showed no signal (Figure 6C). To confirm lack of LC8 association with membrane-bound phospho-nNOS, membranes were immunoprecipitated with LC8 antibody and probed with anti-serine847-P-nNOS antibody. As anticipated, this blotting of membrane fractions did not show any signal (Figure 6C).

8. Cytosolic nNOS isoforms. Like the membrane-bound nNOS, the nature of the cytosolic nNOS was investigated. The cytosolic fraction of the varicosities showed 320, 250 and 155kDa bands when probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody. This band represented the dimer and monomer of nNOS α and dimer of nNOS β (Figure 7A). Subsequently, it was examined whether cytosolic nNOS was bound to calmodulin or not. The cytosolic

extracts were immunoprecipitated with anti-CaM antibody and both the immunoprecipitate and the supernatant were probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody. The cytosolic fraction showed no nNOS in CaM immunoprecipitate (Figure 7B) but showed 320, 250 and 155kDa nNOS bands in the floating fraction (Figure 7B). These results show that all of the cytosolic nNOS bands were CaM lacking. Furthermore, probing the cytosolic CaM-immunoprecipitate and supernatant with anti-serine847-P-nNOS showed three 320, 250 and 155kDa nNOS bands in the floating fraction only, indicating that all the CaM-lacking cytosolic nNOS bands were phosphorylated at serine 847 (Figure 7B).

9. LC8 binding with cytosolic nNOS fractions. To assess whether cytosolic nNOS was associated with LC8, cytosolic extracts were immunoprecipitated with anti-LC8 antibody. Subsequent probing with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody showed 320, 250 and 155kDa bands, indicating that all cytosolic nNOS were associated with LC8 (Figure 7C). Further, cytosolic extracts immunoprecipitated with LC8 antibody and probed with anti-serine847-P-nNOS antibody showed 320, 250 and 155kDa bands (Figure 7C). This confirmed that cytosolic nNOS isoforms, which were all serine847 phosphorylated, were bound to LC8.

Thus, while the membrane-bound 320kDa phosphorylated nNOS α dimer lacked LC8, the cytosolic 320kDa phosphorylated nNOS α was associated with LC8, suggesting that the membrane binding of nNOS influences LC8 binding to nNOS α dimer.

C. Nitric oxide (NO) production by LC8-bound and LC8-lacking nNOS isoforms

As shown in figure 8, immunoprecipitates of varicosity extracts with LC8 showed that the fraction of LC8-bound nNOS produced negligible quantities of nitric oxide in an *in vitro* assay (5 ± 0.58 picomole/30min/mg protein). This nitric oxide production was almost unchanged during preincubation with L-NAME ($p < 0.05$), a drug that inhibits all forms of nNOS enzyme. Nitric oxide was almost not produced when L-arginine was removed from the assay mixture. These studies show that LC8-associated nNOS was catalytically inactive. As shown in Figure 2, these fractions of nNOS represent all isoforms localized to the cytosol of the nerve terminals.

In contrast, precipitates of the IP: LC8 supernatant with anti-calmodulin antibody showed robust NO production (36.67 ± 2.96 pMole/30min/mg protein) (Figure 8). This fraction represented the membrane-bound 320kDa CaM-bound nNOS α dimer that lacked LC8 binding (Figure 3E). The nNOS inhibitors L-NAME significantly inhibited NO production in these samples ($p < 0.01$, paired 2-tailed t test). On the other hand, the floating fraction obtained after CaM-IP of IP: LC8 supernatant showed scant production of nitric oxide in the *in vitro* assay (figure 8), showing that the CaM-lacking nNOS in the LC8-lacking fraction of varicosity extract could not generate nitric oxide. This fraction represented the membrane-bound 320kDa phospho-nNOS α dimer that lacked LC8 binding (Figure 5B).

DISCUSSION

The current studies show that: 1) LC8 is present in the nitrergic nerve terminals of mice gut and is associated with nNOS; 2) LC8 was not associated with CaM-bound nNOS α ; 3) LC8 was associated with CaM-lacking nNOS α and only a component of serine847-P-nNOS α . 4) Membrane-bound nNOS consisted of CaM-bound as well as CaM-lacking, serine847-P-nNOS α dimers; both these components were devoid of LC8 binding; 5) Cytosolic nNOS included nNOS α dimer and monomers and nNOS β dimers, all of which lacked CaM binding and were serine847-phosphorylated; all cytosolic nNOS fractions were associated with LC8; and 6) LC8-bound fractions of nNOS were catalytically inactive, whereas LC8-lacking fractions were both catalytically active and inactive. All the LC8-bound fractions were in the cytosol, whereas the catalytically active LC8-lacking fraction was located only at the membrane of the nerve varicosity.

The presence of LC8 in the nitrergic nerve terminals in mice gut has not been demonstrated before. This is the first study showing the presence of LC8 in the nerve varicosities of the gut and its association with nNOS. The nerve varicosity extracts showed 320kDa, 250kDa and 155kDa nNOS bands that did not react with anti-LC8 antibody in western blots during SDS-PAGE. The 320kDa nNOS dimer band could be visualized due its SDS-resistant nature.(10) The binding between LC8 and nNOS may be weak that was disrupted during denaturing SDS-PAGE. However, association of LC8 and nNOS could be demonstrated in western blots with anti-nNOS antibody using LC8 immunoprecipitated samples of varicosities.

LC8 was associated with three different nNOS bands: 320kDa, 250kDa and 155kDa. The 320kDa and 250kDa nNOS bands represent two different splice variants of nNOS, namely nNOS α and nNOS β ; LC8 was associated with dimers of both nNOS α and nNOS β . 155kDa band represents monomer of nNOS α that was also associated with LC8. Thus, LC8 was associated with both dimers and monomers of nNOS α . These results are similar to those reported before that also showed that LC8 binds with both dimers and monomers of purified nNOS.(17) Moreover, in structural studies, LC8 dimer has been shown to bind with two molecules of nNOS.(12, 22) These observations do not support the view that PIN binding to nNOS dimer destabilizes the dimer and promote monomer formation that was initially suggested as a plausible explanation for the inhibitory effect of PIN on nNOS.(8)

LC8 also bound with nNOS β isoforms. nNOS β isoform in the mouse lacks the first 241 amino acids, at its N-terminal end, that are present in nNOS α .(2) The nNOS β starts with the sequence ¹MRGLGSRDLL¹⁰. Although this sequence is different from the LC8-interacting residues in nNOS α , ²²⁹KDTGIQVDRDLL²⁴⁰, both contain the sequence RDLL that may interact with PIN. The negatively charged aspartic acid (D) of the RDLL sequence in the N-terminal region of nNOS β can form ionic interactions with the positively charged amino acids at the rim of known interacting surface of PIN. Furthermore, the hydrophobic amino acids like leucine (L) can form hydrophobic interactions with the hydrophobic protein-binding surface of PIN. PIN has been predicted to be able to bind to multiple targets with different amino acid sequences using this solvent-exposed hydrophobic surface and positively charged rim.(22) nNOS α and

nNOS β can very likely form heterodimers. This might also explain that during immunoprecipitation with anti-LC8 antibodies, nNOS β might be immunoprecipitated as well. Further studies are needed to identify the exact LC8-binding domain of nNOS β .

One of the interesting findings of this study was the observation that LC8 was not associated with CaM-bound nNOS in the nerve varicosity. This study demonstrated that CaM-bound nNOS was a 320kDa dimer of nNOS α . This result is similar to that reported in an earlier study that also showed that CaM-bound nNOS α dimer was the only active form of nNOS in the nerve terminal.(16) Thus, it appears that LC8 was not associated with active nNOS. The reason why LC8 was not associated with CaM-bound nNOS α is not clear. However, this may be related to the fact that CaM-associated nNOS is associated with varicosity membrane and that this association may be responsible for lack of LC8 binding to CaM bound nNOS.

Another novel finding of this study was that there were two components of serine847-P-nNOS as regards their association with LC8. LC8 was found to be associated with only one of the components of serine847-P-nNOS while the other component was not associated with LC8. Previous studies have shown that CaM-lacking nNOS was serine 847 phosphorylated. Serine847 phosphorylation of nNOS interferes with CaM binding to the molecule and thus renders it inactive with regards to nitric oxide synthesis.(16) Thus, in the nerve varicosities, LC8 was found to be associated with only a fraction of nNOS α .

The reason why LC8 was not bound to a fraction of CaM-lacking, serine847 phosphorylated nNOS α dimer was perplexing. However, it is possible that this finding may be related to membrane binding of the fraction of serine847 phosphorylated nNOS α . Both CaM bound nNOS α and serine847 phosphorylated nNOS α contains a N-terminal PDZ binding domain that is essential for binding to varicosity membrane. PDZ is a structural domain that is found in post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1). The PDZ binding domain of nNOS α allows its binding to the varicosity membrane via PSD95.

To further address these issues, the distribution and the nature of nNOS types in varicosity membranes and cytosol was examined. The membrane and the cytosolic fractions were separated by a combination of osmotic lysis and high velocity-gradient centrifugation and checked for purity. Further, the association of LC8 with the membrane-bound and cytosolic fragments of nNOS was investigated.

The membrane-bound nNOS was found to consist of 320kDa nNOS α dimers of two types: a CaM-bound form and a CaM-lacking, serine847 phosphorylated form. Both these forms were found to lack LC8 association. On the other hand, the cytosolic nNOS α dimer lacked CaM and was serine847 phosphorylated; it was associated with LC8. Cytosolic fractions also contained nNOS α monomer and nNOS β dimer that were also serine847 phosphorylated. All these cytosolic nNOS forms were associated with LC8.

These studies show that PIN was not associated with membrane-bound nNOS α dimer but was associated with only cytosolic nNOS fractions including nNOS α dimers and monomers. These observations suggest that binding of nNOS α dimer to PSD95 in the membrane may dissociate LC8 from the nNOS α dimer. This hypothesis remains to be tested.

Figure 9 summarizes the key findings of the current study. LC8 binds with cytosolic nNOS α dimers and monomers and also nNOS β dimers. All these nNOS forms are phosphorylated at serine847 and are catalytically inactive. LC8 is not associated with either the CaM-bound active or the serine847 phosphorylated membrane-associated nNOS α dimers. We propose that cytosolic LC8-associated inactive nNOS may represent the reserve pool of nNOS α in the nerve terminals and the pool of nNOS α in transit to the varicosity membrane. Association of inactive nNOS α dimer with varicosity membrane leads to dissociation of LC8 from the enzyme. The membrane bound serine847 phosphorylated nNOS α may then get dephosphorylated that can bind with CaM in a calcium dependent fashion. As such, LC8 may be involved in targeting of nNOS to the varicosity membrane and thus play a positive role in maintenance of nitrergic neurotransmission. Future studies are needed to further define the role of LC8 and serine847 phosphorylation and dephosphorylation of membrane-bound nNOS α dimer in nitrergic neurotransmission.

ACKNOWLEDGEMENT

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FIGURE LEGEND

Figure 1. *LC8 is present in nitroergic nerve varicosities.* **A**, Varicosity extracts processed at 4° C showed 3 bands of 320kDa, 250kDa and 155kDa reactive with nNOS₁₄₂₂₋₁₄₃₃. The 320kDa and 250kDa bands were dimers because they were converted to 155kDa and 135kDa monomers on warming the extract at 37° C (Figure 1A, right panel). The 155kDa

signal of nNOS in mouse brain extract (mbe) and rat cerebellum (ce) served as positive controls. **B**, After tricine gel electrophoresis, western blots using anti-LC8 antibody showed a 10kDa band. Rat testes extract, a tissue rich in dynein light chain, served as a positive control. **C**, Nerve varicosities were confirmed by the 42kDa band reactive with synaptophysin antibody.

Figure 2. *LC8 is associated with nNOS in the varicosities.* Immunoprecipitates of varicosities with LC8 antibody probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody at low temperature showed 3 bands at 320, 250 and 155kDas respectively. The IgG control experiment is shown on the left.

Figure 3. *LC8 is not associated with calmodulin (CaM)-bound nNOS.* Varicosity extracts immunoprecipitated with CaM antibody and probed with anti-nNOS₁₄₂₂₋₁₄₃₃ and anti-LC8 antibodies showed a 320kDa nNOS reactive band (**A**) but no LC8 reactive band (**B**). Varicosity extracts immunoprecipitated with LC8 antibody also did not react with anti-CaM antibody (**C**). Appropriate controls are shown. The LC8-lacking fraction obtained in the supernatant after immunoprecipitation with LC8 antibody showed a 320kDa nNOS band during cold SDS-PAGE when probed with an anti-nNOS₁₄₂₂₋₁₄₃₃ antibody and a 155kDa band when the samples were warmed at 37° C (**D**). The supernatant also reacted with anti-CaM antibody and showed a 17kDa band (**E**). A431 cell extract served as a positive control for the calmodulin signal.

Figure 4. *LC8 is associated with CaM-lacking nNOS.* CaM-lacking fraction probed with anti-nNOS and anti-LC8 antibodies showed 3 bands of 320kDa, 250kDa and 155kDa nNOS bands during low temperature SDS-PAGE (**A**). Warm SDS-PAGE of the CaM-lacking fraction showed a 10kDa LC8 reactive band (**B**); rat testes extract was a positive control for LC8. Appropriate IgG controls are also shown.

Figure 5. *LC8 is associated with a component of serine847 phosphorylated nNOS.* Varicosity extracts were immunoprecipitated with LC8 antibody and the immunoprecipitate and the supernatants were probed with anti-serine847-P-nNOS antibody. The LC8-immunoprecipitate showed 3 phospho-nNOS bands (**A**), whereas, the supernatant also showed a 320kDa phospho-nNOS (**B**). Varicosity extract immunoprecipitated with serine847-P-nNOS antibody and probed with anti-LC8 antibody showed a 10kDa signal (**C**). Appropriate IgG controls are shown.

Figure 6. *Membrane bound nNOS isoforms do not bind with LC8.* **A**, The membrane fraction of the varicosities showed only a 320kDa band when probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody; Purity assessment was done by immunodetection of nerve membrane (synaptophysin) and cytosol (LDH) specific markers. **B**, CaM-IP probed with total nNOS antibody showed 320kDa band that did not react with serine847-P-nNOS antibody. The supernatant obtained after CaM-IP also showed a 320kDa band that reacted with serine847-P-nNOS antibody. **C**, LC8-IP probed with total nNOS antibody did not show any signal.

Figure 7. *All cytosolic nNOS isoforms bind with LC8.* **A**, The membrane fraction of the varicosities showed 320, 250 and 155kDa bands when probed with anti-nNOS₁₄₂₂₋₁₄₃₃ antibody; Purity assessment was done by immunodetection of nerve membrane (synaptophysin) and cytosol (LDH) specific markers. **B**, CaM-IP probed with total nNOS antibody showed no signal; the supernatant obtained after CaM-IP showed 320, 250 and 155kDa bands that reacted with total nNOS and serine847-P-nNOS antibodies. **C**, LC8-IP probed with total nNOS and serine847-phospho-nNOS antibodies showed that all cytosolic nNOS isoforms were bound to LC8.

Figure 8. *Quantification of nitric oxide production by LC8-bound and LC-8 lacking fractions of nNOS in mice gut nerve varicosities in vitro.* Note that LC-8 bound nNOS, representing all cytosolic nNOS isoforms, are catalytically inactive. Also note that LC-8 lacking fractions (Supernatant IP-LC8) have two components: a CaM-bound, catalytically active form and a CaM-lacking (Supernatant IP-CaM) catalytically inactive form. Whereas the former represents the CaM-bound nNOS α dimer in the membrane, the later fraction represents membrane-bound CaM-lacking, serine847-phospho-nNOS α dimer. The upper panel shows the standard curve obtained using known concentrations of the nitric oxide donor Nor-3. DAF-2 DA (diaminofluorescein diacetate) conjugate with nitric oxide at 495/515 nm was used to measure levels of nitric oxide.

Figure 9. *Cartoon of gut nerve varicosity summarizing key findings of the current study.* The cytosol of the nerve varicosity has 3 different nNOS isoforms: nNOS α monomer, nNOS α dimer and nNOS β dimer. All these isoforms are phosphorylated at serine847 and

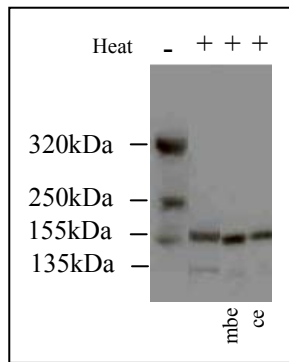
do not bind calmodulin. All these fractions of nNOS in the cytosol are catalytically inactive. The membrane of the nerve varicosity has two types of nNOS: a) catalytically active, CaM-bound nNOS α dimer and (b) catalytically inactive, CaM-lacking, serine847-phospho-nNOS α dimer. LC8 is associated with only cytosolic nNOS isoforms. These findings indicate that LC8 plays a role in storage or transport of different nNOS isoforms in the nerve varicosity. Ca⁺⁺, divalent calcium ion; NO, nitric oxide.

Figure 1.

A.

Varicosity extract

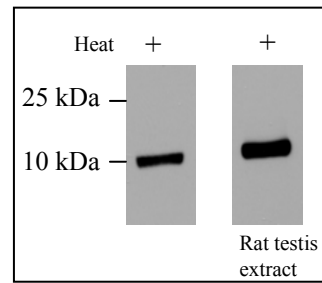
WB: anti-nNOS₁₄₂₂₋₁₄₃₃



B.

Varicosity extract

WB: anti-LC8



C.

Varicosity extract

WB: anti-synaptophysin

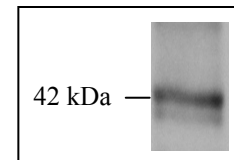


Figure 2.

Varicosity extract

IP: anti-LC8

WB: anti-nNOS₁₄₂₂₋₁₄₃₃

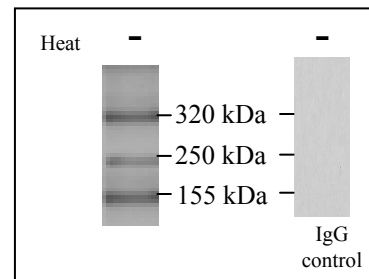


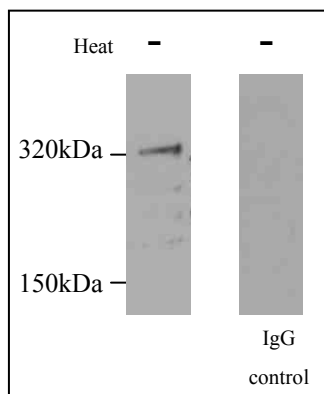
Figure 3.

A.

Varicosity extract

IP: anti-CaM

WB: anti-nNOS₁₄₂₂₋₁₄₃₃

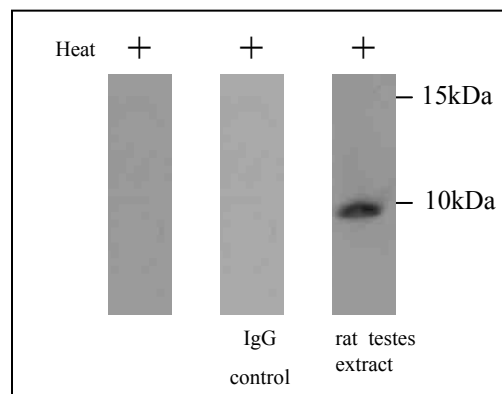


B.

Varicosity extract

IP: anti-CaM

WB: anti-LC8

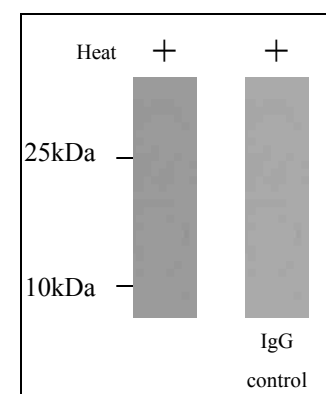


C.

Varicosity extract

IP: anti-LC8

WB: anti-CaM

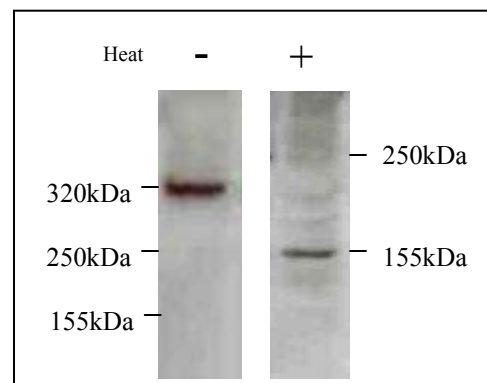


D.

Varicosity extract

IP-LC8 supernatant

WB: anti-nNOS₁₄₂₂₋₁₄₃₃



E.

Varicosity extract

IP-LC8 supernatant

WB: anti-CaM

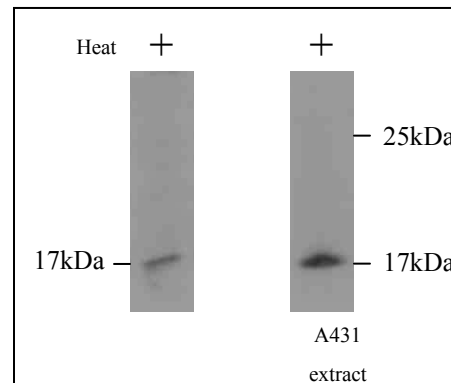
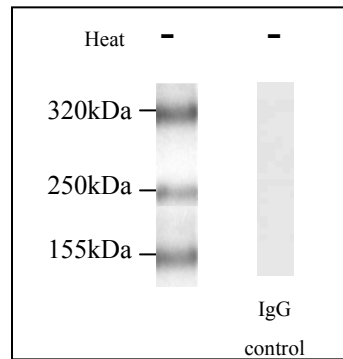


Figure 4.

A.

Varicosity extract
IP-CaM supernatant
WB: anti-nNOS₁₄₂₂₋₁₄₃₃



B.

Varicosity extract
IP-CaM supernatant
WB: anti-LC8

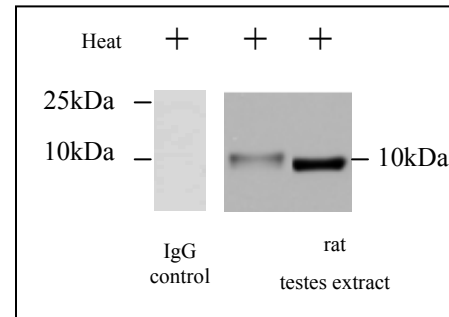


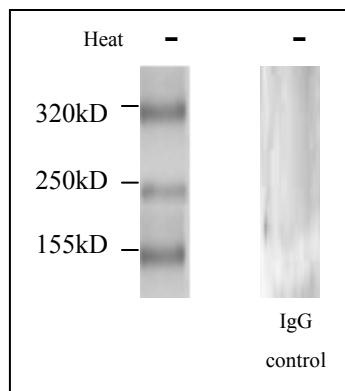
Figure 5.

A.

Varicosity extract

IP: anti-LC8

WB: anti-serine847-phospho-nNOS

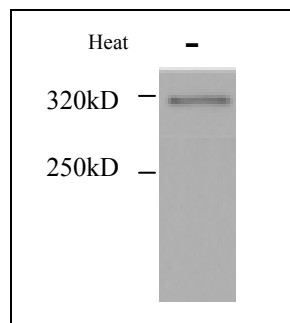


B.

Varicosity extract

IP-LC8 supernatant

WB: anti-serine847-phospho-nNOS



C.

Varicosity extract

IP: anti-Serine847-phospho-nNOS

WB: anti-LC8

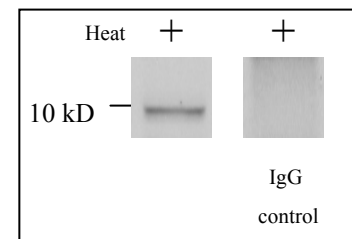


Figure 6.

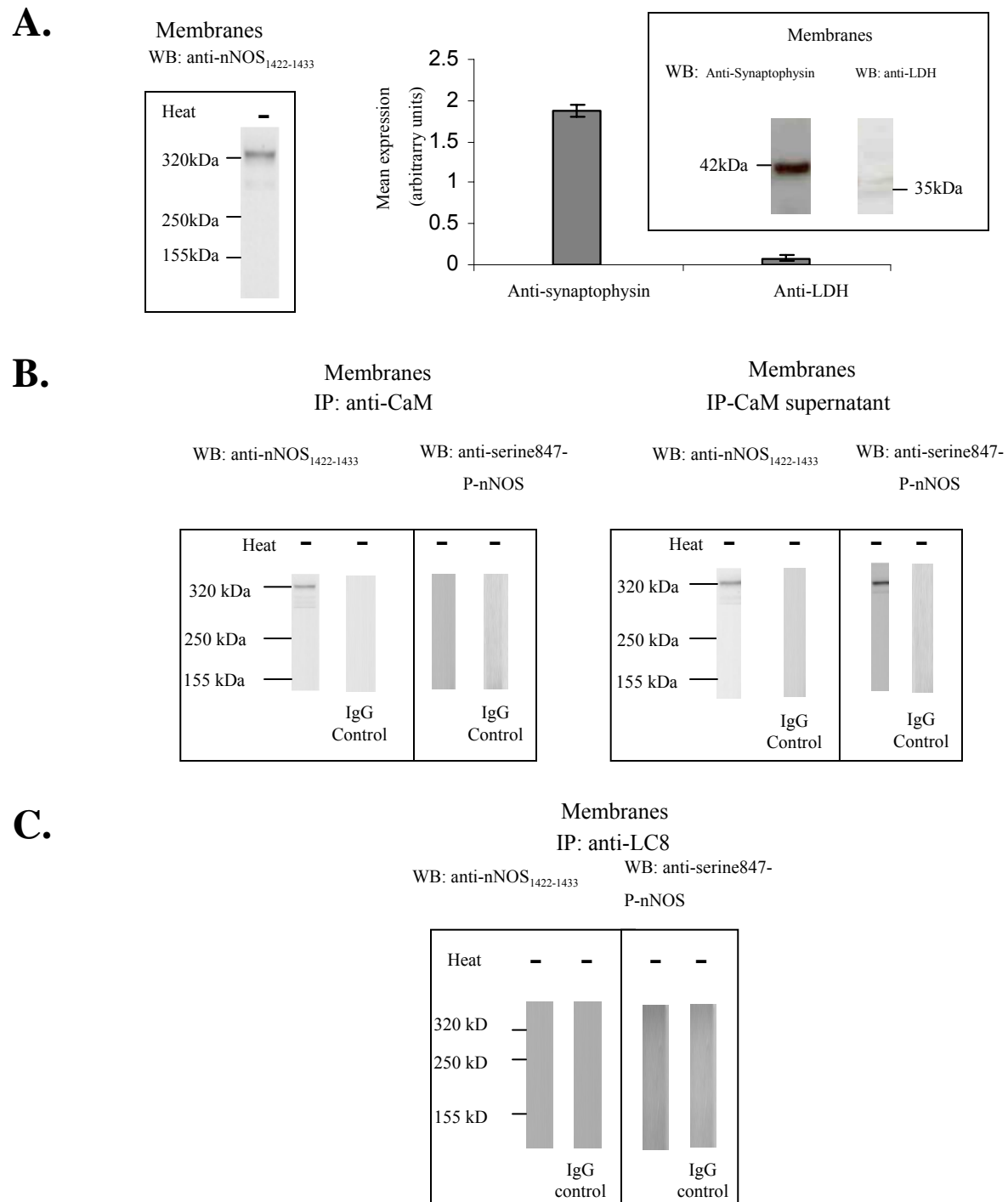


Figure 7.

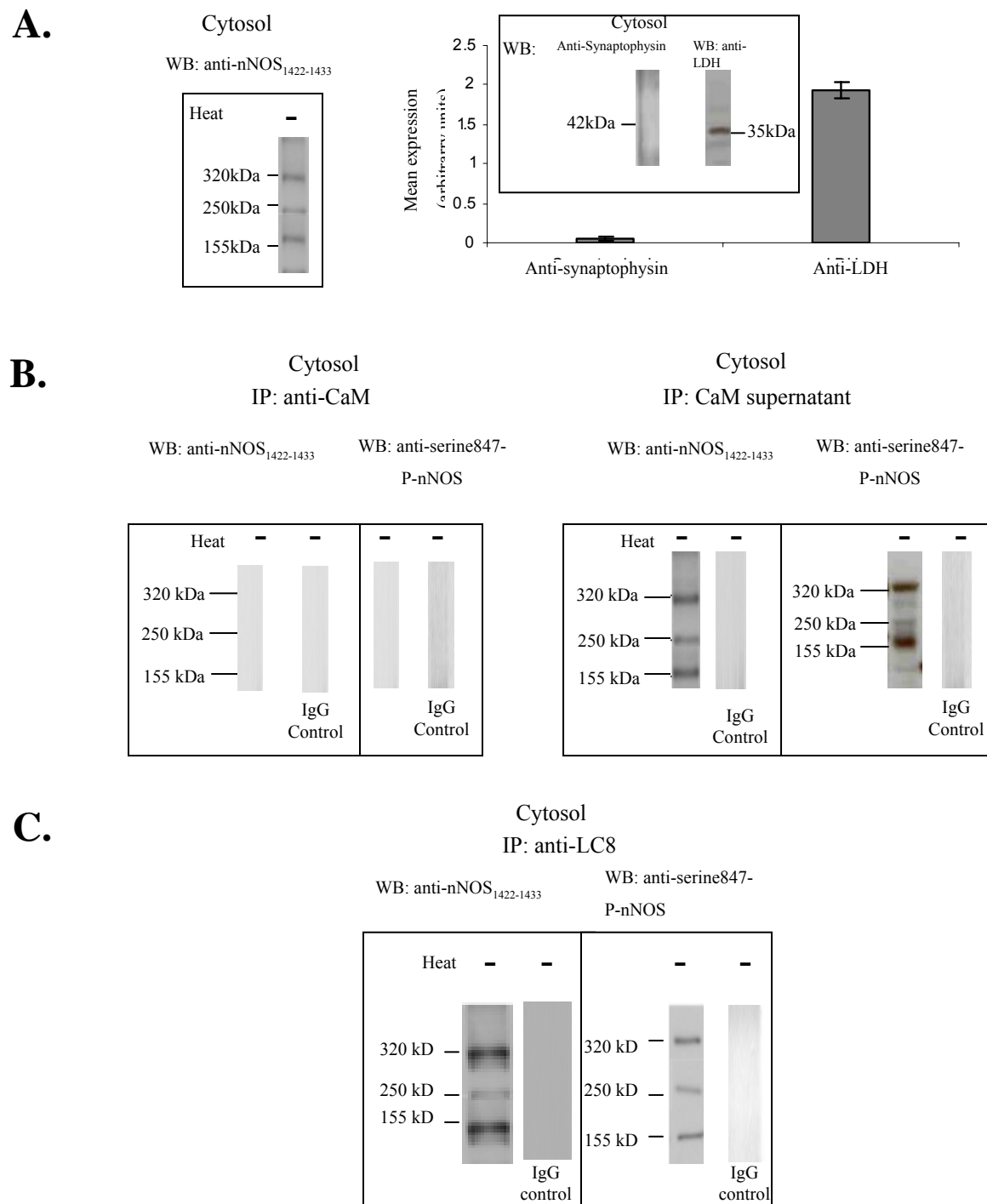


Figure 8.

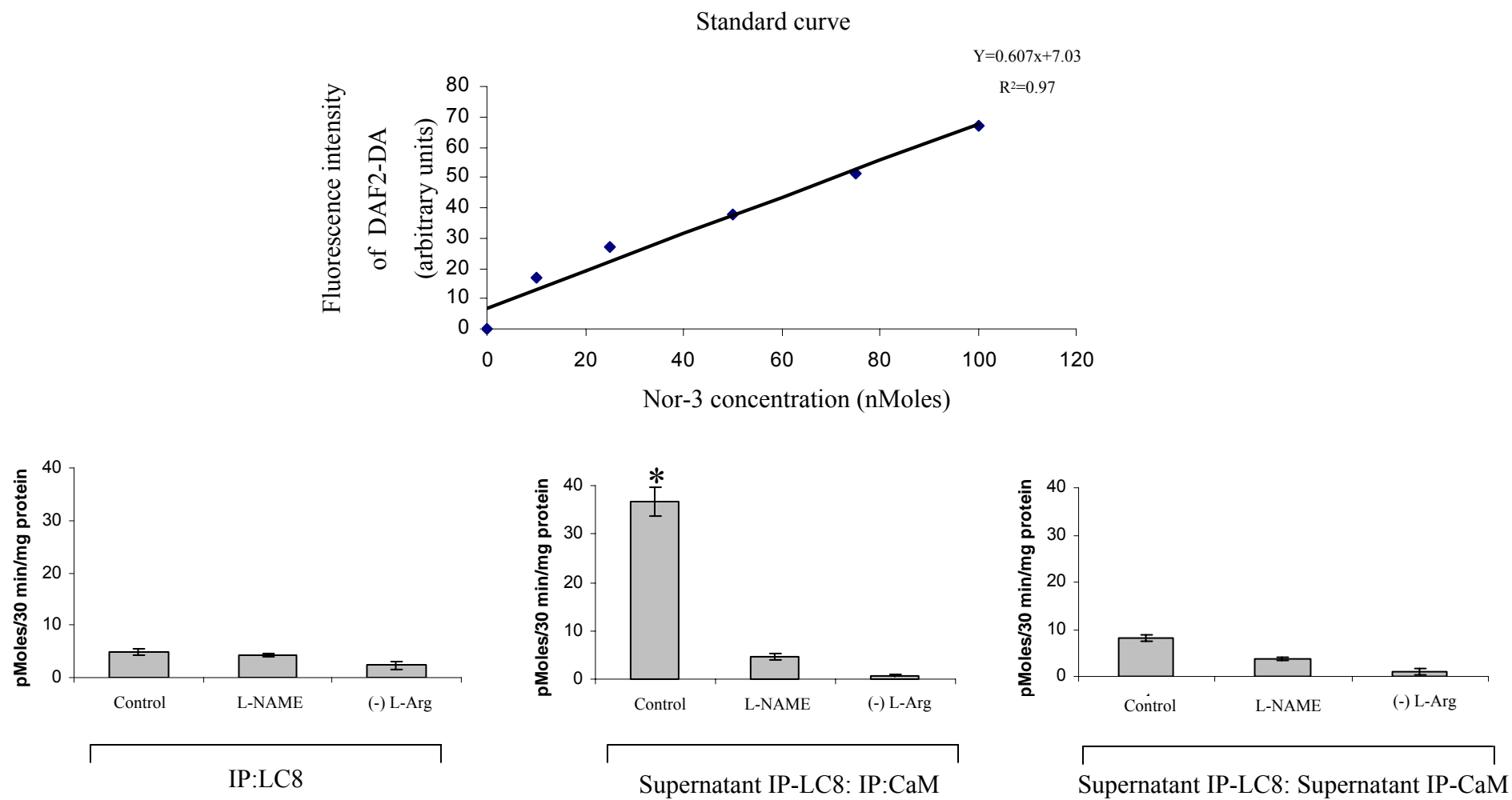


Figure 9.

