

UBIQUITINATION AND DEGRADATION OF
NEURONAL NO-SYNTASE IN VITRO: DIMER STABILIZATION PROTECTS
THE ENZYME FROM PROTEOLYSIS*

Anwar Y. Dunbar[#]
Yasuhiko Kamada
Gary J. Jenkins
Ezra R. Lowe[†]
Scott S. Billecke
Yoichi Osawa[†]

From the Department of Pharmacology,
The University of Michigan Medical School,
Ann Arbor, Michigan 48109

MOL #000125

Running Title: Proteolysis of neuronal NOS

Corresponding Author:

Yoichi Osawa
Department of Pharmacology
The University of Michigan Medical School
1301 Medical Science Research Building III
Ann Arbor, MI 48109-0632
Tel: (734) 936-5797
Fax: (734) 763-4450
Email: osawa@umich.edu

Text 26 pages

Tables 0

Figures 7

References 24

Abstract 197 words

Introduction 427 words

Discussion 654 words

The abbreviations used are: NOS, nitric oxide synthase; nNOS, neuronal NOS; apo-nNOS, heme-deficient nNOS; holo-nNOS, heme-sufficient nNOS; SDS-PAGE, SDS polyacrylamide gel electrophoresis; MG132, Cbz-leucine-leucine-leucinal; BH₄, (6R)-5,6,7,8-Tetrahydro-L-biopterin; 7-NI, 7-nitroindazole; NNA, N^G-nitro-L-arginine.

MOL #000125

ABSTRACT

It is established that neuronal NO synthase (nNOS) is ubiquitinated and proteasomally degraded. The metabolism-based inactivation of nNOS as well as the inhibition of hsp90-based chaperones, which are known to regulate nNOS, both lead to enhanced proteasomal degradation of nNOS. The mechanism of this selective proteolytic degradation, or in essence how the nNOS becomes labilized and recognized for ubiquitination and subsequent degradation, has not been determined. In the current study, we used a crude preparation of reticulocyte proteins, which contains ubiquitin conjugating enzymes and the proteasome, to determine how nNOS is labilized. We found that the inactive monomeric heme-deficient apo-nNOS is rapidly degraded *in vitro*, consistent with the finding that both metabolism-based inactivation and inhibition of hsp90-based chaperones cause the formation of apo-nNOS and enhance its degradation *in vivo*. In the current study we discovered that destabilization of the dimeric nNOS, as determined by measuring the SDS-resistant dimer, is sufficient to trigger ubiquitin-proteasomal degradation. Treatment of nNOS with N^G-nitro-L-arginine or 7-nitroindazole led to stabilization of the dimeric nNOS and decreased proteasomal degradation of the enzyme, consistent with that observed in cells. Thus, it appears that the dimeric structure is a major determinant of nNOS stability and proteolysis.

MOL #000125

INTRODUCTION

Nitric oxide synthase (NOS) is a flavin, heme, and tetrahydrobiopterin -containing enzyme that catalyzes, in the presence of NADPH and oxygen, the metabolism of L-arginine to nitric oxide, which is an important cellular signaling molecule. NOS is active as a homodimer when bound with calcium/calmodulin. NOS is highly regulated by a variety of mechanisms, including selective proteolytic degradation (Osawa et al., 2003). One pathway for degradation involves ubiquitination and proteasomal degradation, which is affected by a variety of factors including hsp90-based chaperones (Bender et al., 1999; Jiang et al., 2003), caveolin (Felley-Bosco et al., 2000), and NOS inactivators (Noguchi et al., 2000). The effect of inactivators may be of particular importance in the development of inhibitors for clinical use (Metz et al., 2003).

In the case of the neuronal isoform of NOS (nNOS), metabolism-based inactivators, which act by formation of reactive intermediates that covalently alter the enzyme, accelerate the degradation of nNOS in cells; whereas reversible inhibitors, such as 7-nitroindazole and N^G-nitro-L-arginine, stabilize or have no effect on the protein (Noguchi et al., 2000). Thus, the molecular trigger for proteolysis is not the loss of enzyme activity per se but is dictated by the mechanism of inhibition. Consistent with these findings, treatment of rats with metabolism-based inactivators causes a decrease in nNOS activity and content (Nakatsuka et al., 1998). The exact molecular mechanism for these effects is unknown, although the degradation of nNOS in cells is known to involve ubiquitination and proteasomal degradation (Bender et al., 2000a;Noguchi et al., 2000). The metabolism-based inactivators are known to cause the alteration and destruction of the heme prosthetic group *in vitro* and *in vivo* (Jianmongkol et al., 2000;Vuletich et al., 2002) and lead to formation of the heme-deficient apo-nNOS. Interestingly, the monomeric heme-deficient form was found to be preferentially ubiquitinated over that of the dimeric fully active holo-nNOS (Bender et al., 2000a). In the current study we used an *in vitro* system containing partially purified reticulocyte proteins to model the cellular degradation of nNOS so that the nature of the molecular trigger for proteasomal degradation of

MOL #000125

nNOS could be examined. We found that an *in vitro* system containing fraction II, which is the DE52-retained fraction of reticulocyte lysate proteins, can faithfully reproduce the regulated ubiquitination and proteasomal degradation of nNOS. Although it is established that the heme-deficient apo-nNOS is preferentially ubiquitinated over the heme-containing holoenzyme (Bender et al., 2000a), in the current study we found that heme loss from the holoenzyme is likely not a prerequisite for degradation and that dimer instability is a molecular trigger for ubiquitination and proteasomal degradation.

MOL #000125

MATERIALS AND METHODS

Materials

(6R)-5,6,7,8-Tetrahydro-L-biopterin (BH₄) was purchased from Dr. Schirck's Laboratory (Jona, Switzerland). L-arginine, FAD, myoglobin (horse heart), ATP, ubiquitin (bovine), EGTA, calpain inhibitor peptide, N-nitro-L-arginine, MgCl₂, creatine phosphokinase, hexokinase, and 2-deoxy-D-glucose were purchased from Sigma. The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from Transduction Laboratories (Lexington, KY). ¹²⁵I-labeled antibody against rabbit IgG was purchased from Perkin Elmer NEN (Boston, MA). Cbz-leucine-leucine-leucinal (MG132) and 7-nitroindazole were purchased from BIOMOL (Plymouth Meeting, PA). 3-(5-Fluoro-3-indolyl)-2-mercapto-(Z)-2-propenoic Acid (PD151746) was from Calbiochem. Ubiquitin aldehyde was from Alexis Biochemicals (San Diego, CA). Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). DE52 was purchased from Whatman Inc. (Fairfield, NJ).

Methods

Expression and purification of holo-nNOS and apo-nNOS - nNOS was overexpressed in Sf9 insect cells as previously described (Bender et al., 1999). To express holo-nNOS, oxyhemoglobin (25 μM) was added as a source of heme during the last 24 hr of expression. Cells were harvested and suspended in 1 volume of 10 mM Hepes, pH 7.5, containing 320 mM sucrose, 100 μM EDTA, 0.1 mM dithiothreitol, 10 μg/ml trypsin inhibitor, 1.0 μM leupeptin, 2 μg/ml of aprotinin, 6 mM phenylmethanesulphonyl fluoride, and 10 μM BH₄, and the suspended cells were ruptured by Dounce homogenization. Lysates from infected Sf9 cells (8 x 10⁹) were centrifuged at 100,000 x g for 1 h. The supernatant fraction was loaded onto a 2'5'-ADP Sepharose column (20 ml) and the nNOS was affinity purified as described (Roman et al., 1995), except that 10 mM 2' AMP in high salt buffer was used to elute the protein. The nNOS-containing fraction was concentrated with the use of a Centriplus YM-10 concentrator (Amicon,

MOL #000125

10,000 MWCO) to 10 ml and loaded onto a Sephacryl S-300 HR gel filtration column (2.6 x 100 cm, Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5 μ M BH₄. The proteins were eluted at a flow rate of 1.0 ml/min and 1.0 ml-fractions were collected and analyzed for protein content and NOS activity. The fractions containing NOS activity were pooled and supplemented with 10 μ M BH₄ and concentrated with the use of a Centriplus YM-10 concentrator. This Sephacryl-purified nNOS preparation had a specific activity of approximately 1000 nmol/min/mg of protein and was stored at -80 °C. To prepare apo-nNOS, the procedure was the same as that for holo-nNOS, except that oxyhemoglobin was omitted during expression and BH₄ was not added during purification. The specific activity of the apo-nNOS preparation was approximately 25 nmol/min/mg of protein.

Treatment of apo-nNOS and holo-nNOS with fraction II – Fraction II was prepared from rabbit reticulocyte lysates as previously described (Hershko et al., 1983), except that there were two successive DE52 chromatography steps. Each preparation of fraction II was tested with carboxymethylated BSA as a positive control. The nNOS preparations (2 μ g) were incubated at 37°C in a total volume of 120 μ l of 50 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol, 50 μ M ubiquitin, an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, 5 mM MgCl₂, and 10 units/ml creatine phosphokinase), and 2 mg/ml of fraction II. At indicated times, the samples were quenched with 25 μ l of sample buffer containing 5% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.02% bromophenol blue in 125 mM Tris-HCl, pH 6.8. The samples were boiled for 3 min and an aliquot (25 μ l) was submitted to 6% SDS-PAGE (10 x 8 cm). Proteins were then transferred to nitrocellulose membranes (0.2 μ m, BioRad) and probed with 0.1% anti-nNOS. The immunoblots were then incubated a second time with ¹²⁵I-conjugated goat anti-rabbit IgGs to visualize the immunoreactive bands. The membranes were dried and exposed to X-OMAT film for 1 h at -80°C. The bands corresponding to nNOS were excised and the radioactivity quantified by the use of a gamma counter. This method was quantitative up to

MOL #000125

0.5 μg of nNOS with a linear relationship between the amount of nNOS and radioactivity ($r^2 = 0.99$).

In studies where the SDS-resistant dimer was measured, the reaction mixture was quenched with sample buffer supplemented with 100 μM BH_4 and 100 μM L-arginine. The samples were kept on ice prior to loading for analysis by SDS-PAGE as described above. This method has previously been described by Klatt *et al.* (Klatt et al., 1995) to prevent the dissociation of nNOS dimers prior to and during electrophoresis.

Heme measurements - The heme present in nNOS was assessed by measuring the ferrous-CO complex. Since fraction II contained compounds that exhibit absorption maxima in the 420-nm region, we partially purified the reaction mixtures for nNOS. Reaction mixtures (85 μg of nNOS protein) were loaded onto a 2'5'-ADP Sepharose column (2.0 cm x 0.8 cm) and purified as described above. The ferrous-CO complex was measured as a difference spectrum and quantified with an extinction coefficient of 91 mM^{-1} , as previously described (McMillan et al., 1992). The protein was measured by Coomassie Plus Protein Reagent from Pierce (BioRad) with the use of bovine serum albumin as standard. The heme content per protein of nNOS was determined to be 0.56 ± 0.06 mole of P450/mole monomer of nNOS (mean \pm SD, $n=3$). The FAD content per protein was measured by HPLC analysis with fluorescence detection on 2.4 μg of nNOS (Klatt et al., 1996). The assay was linear from 1-30 pmol of FAD ($r^2=0.998$). The FAD content is 0.77 ± 0.11 mole/mole monomer of nNOS (mean \pm SD, $n=4$). Of course, these values are based on the crude estimation of the protein amounts and thus the true molar value of the protein is suspect. However, comparison of the heme content to that of the FAD content removes the error due to the protein measurement and indicates that the heme content is approximately 0.7 mol/mol.

MOL #000125

RESULTS

Heme-deficient apo-nNOS is degraded by the proteasome in vitro — Fraction II comprises the DE52-bound proteins from reticulocyte lysates and includes a variety of ubiquitin ligases as well as the proteasome. As shown in Fig. 1, treatment of purified heme-deficient apo-nNOS with fraction II, ATP, and ubiquitin caused the loss of nNOS protein over a 90-min period (*lanes 1-4*). The loss of protein was not accompanied by the appearance of proteolytic fragments. Although this may be due to a lack of immune recognition of the peptides, the loss of protein without large peptide fragments is consistent with degradation by the proteasome. As shown in Fig. 1, when fraction II was boiled before addition to the reaction mixture, there was no loss of apo-nNOS, indicating that there are heat-labile factors responsible for the loss of nNOS (*lanes 5-8*). The amount of nNOS on these blots was quantified by directly counting the ¹²⁵I-labeled secondary antibody (Fig. 2A). There was an approximately 75% loss of apo-nNOS over 90 min (*closed circles*) compared to less than 10% loss when boiled fraction II was used (*closed squares*). The omission of ATP decreased the amount of apo-nNOS degraded, indicating an ATP-dependent process (*open circles*). As shown in Fig. 2B, MG132, a proteasome inhibitor, protected apo-nNOS from degradation in a concentration-dependent manner. Since the omission of ATP or addition of MG132 did not completely abolish the loss of apo-nNOS, other proteases or even other processes such as aggregation account for approximately 25% of the loss of nNOS over a 90-min incubation time. Calpain is known to proteolytically cleave nNOS (Laine et al., 1998); however, as shown in the Fig. 2B *inset*, calpain inhibitor peptide (*CIP*), PD151746 (*PD*), and EGTA (*EGTA*) at concentrations that have been reported to inhibit calpain (Laine et al., 1998; Squier et al., 1999; Walker et al., 2001) did not have an effect on the loss of nNOS under these conditions. Thus, the predominant pathway observed here is the proteasomal degradation of nNOS.

We further examined the role of ubiquitin in the degradation process and found that at the high concentration of fraction II (2 mg/ml) there was enough endogenous ubiquitin present to

MOL #000125

support ubiquitination ligation reactions that could easily be detected by an anti-ubiquitin antibody (data not shown). Therefore, we examined the degradation reaction at a lower concentration of fraction II (0.2 mg/ml) even though the proteolysis was approximately 50%, and thus not as extensive (Fig. 3, *condition 2*). In the absence of exogenous ubiquitin, the amount of nNOS decreased by 25% (*condition 1*), which is comparable to that observed with 10 μ M MG132 under these conditions. The addition of ubiquitin aldehyde at low concentrations (*condition 3*) is known to inhibit deubiquitinating enzymes (Shaeffer et al., 1996) but had only a small effect on the loss of nNOS at 90 min. At 20 min of incubation, we observed a 20% greater loss of nNOS due to 0.7 μ M ubiquitin aldehyde compared to control samples (data not shown) that suggests that there may be a small enhancement in degradation when ubiquitin conjugates are stabilized. The low concentration of ubiquitin aldehyde gave an enhanced level of ubiquitination of cellular proteins as visualized by an anti-ubiquitin antibody, whereas ubiquitin aldehyde at a higher concentration (15 μ M) decreased the overall level of ubiquitin protein conjugates (data not shown). This higher concentration of ubiquitin aldehyde protected nNOS from degradation (*condition 4*), further confirming the role of ubiquitin in the proteasomal degradation of nNOS.

Studies on nNOS dimer stability and proteolytic degradation – To further investigate the nature of the nNOS that triggers its degradation, we studied the degradation of heme-sufficient holo-nNOS in contrast to that performed above with heme-deficient apo-nNOS. The inactive monomeric heme-deficient apo-nNOS is known to be preferentially ubiquitinated whereas the active dimeric holo-nNOS is not ubiquitinated (Bender et al., 2000a) and thus, we expected that holo-nNOS would not be degraded. To our surprise holo-nNOS was degraded (Fig. 4, *open squares*), albeit not as extensively as apo-nNOS above (compare with Fig. 2A, *closed circles*). The loss of holo-nNOS was largely attenuated by MG132 indicating degradation was predominantly by the proteasome (*closed squares*), highly similar to that found for apo-nNOS. To further understand why holo-nNOS was degraded, we examined the samples by low temperature SDS-PAGE so that the SDS-resistant dimeric form of nNOS could be measured

MOL #000125

(Klatt et al., 1995). nNOS forms a very tight dimer that is resistant to SDS at low temperatures. By keeping the samples on ice and running the SDS-PAGE with a cooling unit, the stable dimeric species can be visualized. This assay is not a measure of the dimeric content under native conditions but is a measure of the amount of stable dimer that is not dissociated by SDS and thus underestimates the total dimeric content. Moreover, it is likely that the transfer efficiency of the dimer is lower than that of the monomer further leading to the underestimation of dimeric content. As shown in Fig. 5A, the holo-nNOS exists in part as a SDS-resistant dimer (*lane 1, nNOS dimer*). This dimeric form was unstable and was converted to the band corresponding to the monomeric form over the 90-min incubation period (*lanes 2-4*). The presence of MG132 did not prevent the loss of the SDS-resistant dimeric form (*lanes 5-8*). The bands representing the dimeric as well as the monomeric nNOS were quantified and the results are plotted in Fig. 5B. Clearly, the presence of MG132 had no effect on the loss of dimeric nNOS (*cf. closed squares with closed circles*). Interestingly, the band corresponding to the monomeric form of nNOS accumulated during the incubation period when MG132 was present (*cf. open circles with open squares*), indicating that the monomeric form is proteasomally degraded. There appears to be little loss of the band corresponding to the monomeric form in the absence of MG132 (*open squares*), most likely due to replenishment of the monomeric nNOS pool from the monomerization of the dimeric nNOS at a rate similar to that of proteasomal degradation.

To further confirm the notion that the monomeric form is preferentially degraded, we sought to stabilize the dimeric nNOS. As shown in Fig. 6A, the addition of 7-nitroindazole to the reaction mixture stabilized the SDS-resistant dimeric form of nNOS (*cf. closed squares with closed circles*). This stabilization of the dimer prevented the replenishment of the monomeric pool and resulted in a nearly complete loss of the monomeric form (*open circles*). To further confirm that stabilization of the dimer had an effect on the degradation of holo-nNOS, we measured the loss of the total nNOS, not differentiating among the dimeric and monomeric species, by regular SDS-PAGE analysis (Fig. 6B). The loss of nNOS was greatly diminished in

MOL #000125

the presence of 10 μM 7-nitroindazole (*cf. open squares with closed squares*). Highly similar results were obtained for 10 μM N^{G} -nitro-L-arginine (Fig. 6C and 6D). Moreover, N^{G} -nitro-L-arginine had no protective effect on the degradation of apo-nNOS (Fig. 6D, *closed triangle*), indicating specificity of action for the holoenzyme. Thus, it appears that destabilization of the dimeric nNOS, most likely to the inactive monomeric state, is the trigger that renders nNOS susceptible to selective ubiquitination and proteasomal degradation. As shown in Fig. 7, to support further the notion that destabilization and not heme stripping is important, we found that holo-nNOS incubated with fraction II for 60 min in the presence of 10 μM MG132 still retains approximately 90% of the original ferrous-CO complex (*cf. spectrum 1 with spectrum 2*). Thus, there is very little apo-nNOS formed from holo-nNOS during the incubation with fraction II. Based on the heme content of 0.7 mol/mol for the purified nNOS preparation used, we cannot exclude the possibility that some of the degradation is due to the apo-nNOS present. However taken together, the lack of change in the P450 values and the nearly complete protection from proteasomal degradation by 7-nitroindazole and N^{G} -nitro-L-arginine, which are known to bind holo-nNOS, suggest that destabilization of the dimer and not heme loss per se is a critical determinant in the proteasomal degradation of nNOS.

MOL #000125

DISCUSSION

NOS is ubiquitinated and proteasomally degraded *in vivo* (Noguchi et al., 2000;Bender et al., 2000a). In the current study, we used an *in vitro* system to examine the ‘trigger’ for this proteasomal degradation. The *in vitro* system utilizes a reticulocyte protein preparation, or fraction II, that recapitulates the ubiquitin- and ATP-dependent proteasomal degradation of nNOS. With the use of this system, we found that the inactive, monomeric, heme-deficient apo-nNOS is readily degraded, consistent with the finding that apo-nNOS is preferentially ubiquitinated over that of the heme-containing holo-enzyme (Bender et al., 2000a). This is also consistent with the notion that metabolism-based inactivators, which can covalently alter and destroy the heme prosthetic group, as well as inhibitors to hsp90, which prevent heme insertion into apo-nNOS, both enhance the proteasomal degradation of nNOS (Noguchi et al., 2000;Bender et al., 1999;Billecke et al., 2002). Although a previous study suggested that heme loss was a determinant of ubiquitination (Bender et al., 2000a), our current study indicates that heme loss per se is likely not the only trigger for degradation as described below.

Although dimeric holo-nNOS is not ubiquitinated (Bender et al., 2000a), we unexpectedly found that holo-nNOS was proteasomally degraded, albeit at a slightly slower rate than for apo-nNOS. Further studies measuring the stability of the holo-nNOS by low temperature SDS-PAGE indicated that the dimeric form was unstable during the incubation with fraction II. Inhibition of the proteasome did not affect the stability of the dimeric nNOS although it prevented the loss of the destabilized nNOS. The reversible NOS inhibitors, 7-NI and NNA, stabilize the dimeric nNOS and protect nNOS from proteasomal degradation. As expected, there was no effect on apo-nNOS, which cannot dimerize without heme and does not bind 7-NI or NNA. The finding that 7-NI and NNA protect against degradation is consistent with the observation of increased levels of nNOS in cells treated with these agents (Noguchi et al., 2000) and in rats (Nakatsuka et al., 1998) and further validates the *in vitro* system.

Taken together, it appears that destabilization of the dimeric form of nNOS is a trigger for degradation. This destabilization of the dimeric form could lead to formation of a more loosely

MOL #000125

associated dimer (Bender et al., 2000b), a heme-containing monomeric form, or heme-deficient apo-nNOS. It appears that nearly all the heme remains in the destabilized nNOS during incubations with fraction II in the presence of MG132, as evident by the presence of ferrous-heme-CO complex of nNOS. This clearly indicates that only a small amount of apo-nNOS is formed and thus, heme loss appears not to be a prerequisite for degradation. One form of loosely associated dimer, which is prepared by *in vitro* reconstitution of apo-nNOS with heme (Bender et al., 2000b), was tested and found to be rapidly degraded (data not shown). The degradation of the heme-containing monomeric species has not been investigated. Although the exact nature of the substrate for ubiquitination is not known at present, it is clear that destabilization of the dimer is an important event and we speculate that a degradation signal is present in the dimer interface that is sterically blocked in the stable dimeric state. In support of this notion, heterodimerization of transcription factors, MAT α 2 and MAT α 1, is known to decrease the ubiquitin-proteasomal degradation of both factors (Johnson et al., 1998). This mutual stabilization is thought to be due to steric factors that mask the degradation signal. Alternatively, the destabilization of the dimeric nNOS leads to a relaxation of the structural constraints, rendering the protein more flexible and/or disordered. This relaxation of the protein may be sufficient to target the protein for degradation. It is noteworthy that destabilization of dimeric nNOS also leads to enhanced susceptibility to phosphorylation by protein kinase C (Okada, 1998) and hydrolysis by trypsin (Panda et al., 2002). In the case of proteasomal degradation, the regulated removal of inactive monomeric nNOS over that of the active dimeric species would appear to be beneficial.

MOL #000125

ACKNOWLEDGMENTS

We thank Solomon Snyder for providing the nNOS cDNA used in this work.

MOL #000125

REFERENCES

Bender AT, Demady DR, and Osawa Y (2000a) Ubiquitination of neuronal nitric oxide synthase *in vitro* and *in vivo*. *J. Biol. Chem.* **275**:17407-17411.

Bender AT, Nakatsuka M, and Osawa Y (2000b) Heme insertion, assembly, and activation of apo-neuronal nitric-oxide synthase *in vitro*. *J. Biol. Chem.* **275**:26018-26023.

Bender AT, Silverstein AM, Demady DR, Kanelakis KC, Noguchi S, Pratt WB, and Osawa Y (1999) Neuronal nitric oxide synthase is regulated by the hsp90-based chaperone system *in vivo*. *J. Biol. Chem.* **274**:1472-1478.

Billecke SS, Bender AT, Kanelakis KC, Murphy PJ, Lowe ER, Kamada Y, Pratt WB, and Osawa Y (2002) hsp90 is required for heme binding and activation of apo-neuronal nitric-oxide synthase: geldanamycin-mediated oxidant generation is unrelated to any action of hsp90. *J. Biol. Chem.* **277**:20504-20509.

Felley-Bosco E, Bender FC, Courjault-Gautier F, Bron C, and Quest AF (2000) Caveolin-1 down-regulates inducible nitric oxide synthase via the proteasome pathway in human colon carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **97**:14334-14339.

Hershko A, Heller H, Elias S, and Ciechanover A (1983) Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J. Biol. Chem.* **258**:8206-8214.

Jiang J, Cyr D, Babbitt RW, Sessa WC, and Patterson C (2003) Chaperone-dependent Regulation of Endothelial Nitric-oxide Synthase Intracellular Trafficking by the Co-chaperone/Ubiquitin Ligase CHIP. *J. Biol. Chem.* **278**:49332-49341.

MOL #000125

Jianmongkol S, Vuletich JL, Bender AT, Demady DR, and Osawa Y (2000) Aminoguanidine-mediated inactivation and alteration of neuronal nitric oxide synthase. *J. Biol. Chem.* **275**:13370-13376.

Johnson PR, Swanson R, Rakhilina L, and Hochstrasser M (1998) Degradation signal masking by heterodimerization of MATalpha2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell* **94**:217-227.

Klatt P, Schmidt K, Lehner D, Glatter O, Bachinger HP, and Mayer B (1995) Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J.* **14**:3687-3695.

Klatt P, Schmidt K, Werner ER and Mayer B (1996) Determination of nitric oxide synthase cofactors: heme, FAD, FMN, and tetrahydrobiopterin. *Methods Enzymol.* **268**:358-365.

Laine R and Ortiz de Montellano PR (1998) Neuronal nitric oxide synthase isoforms alpha and mu are closely related calpain-sensitive proteins. *Mol. Pharmacol.* **54**:305-312.

McMillan K, Bredt DS, Hirsch DJ, Snyder SH, Clark JE and Masters BSS (1992) Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 11141-11145.

Mete A and Connolly S (2003) Inhibitors of the NOS enzymes: a patent review. [148 refs]. *Idrugs* **6**:57-65.

Nakatsuka M, Nakatsuka K, and Osawa Y (1998) Metabolism-based inactivation of penile nitric oxide synthase activity by guanabenz. *Drug Metab. Dispos.* **26**:497-501.

Noguchi S, Jianmongkol S, Bender AT, Kamada Y, Demady DR, and Osawa Y (2000) Guanabenz-mediated inactivation and enhanced proteolytic degradation of neuronal nitric oxide synthase. *J. Biol. Chem.* **275**:2376-2380.

MOL #000125

Okada D (1998) Tetrahydrobiopterin-dependent stabilization of neuronal nitric oxide synthase dimer reduces susceptibility to phosphorylation by protein kinase C in vitro. *FEBS Lett.* **434**:261-264.

Osawa Y, Lowe ER, Everett AC, Dunbar AY, and Billecke SS (2003) Proteolytic degradation of nitric oxide synthase: effect of inhibitors and role of hsp90-based chaperones. *J. Pharmacol. Exp. Ther.* **304**:1-5.

Panda K, Rosenfeld RJ, Ghosh S, Meade AL, Getzoff ED, and Stuehr DJ (2002) Distinct Dimer Interaction and Regulation in Nitric-oxide Synthase Types I, II, and III. *J. Biol. Chem.* **277**:31020-31030.

Roman LJ, Sheta EA, Martasek P, Gross SS, Liu Q, and Masters BS (1995) High-level expression of functional rat neuronal nitric oxide synthase in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* **92**:8428-8432.

Shaeffer JR and Cohen RE (1996) Differential effects of ubiquitin aldehyde on ubiquitin and ATP-dependent protein degradation. *Biochemistry* **35**:10886-10893.

Squier MK, Sehnert AJ, Sellins KS, Malkinson AM, Takano E, and Cohen JJ (1999) Calpain and calpastatin regulate neutrophil apoptosis. *J. Cell Physiol.* **178**:311-319.

Vuletich JL, Lowe ER, Jianmongkol S, Kamada Y, Kent UM, Bender AT, Demady DR, Hollenberg PF, and Osawa Y (2002) Alteration of the heme prosthetic group of neuronal nitric-oxide synthase during inactivation by N(G)-amino-L-arginine in vitro and in vivo. *Mol. Pharmacol.* **62**:110-118.

Walker G, Pfeilschifter J, Otten U, and Kunz D (2001) Proteolytic cleavage of inducible nitric oxide synthase (iNOS) by calpain I. *Biochem. Biophys. Acta* **1568**:216-224.

MOL #000125

FOOTNOTES

* This investigation was supported by National Institutes of Health Grants ES08365

‡ Trainee under Pharmacological Sciences Training Program GM07767 from the National Institutes of Health.

Trainee under Minority Supplement to ES08365

† Established Investigator of the American Heart Association.

† Correspondence may be addressed to the senior author at the Department of Pharmacology, The University of Michigan Medical School, Medical Science Research Building III, Ann Arbor, Michigan 48109-0632; FAX: (734) 763-4450.

LEGENDS FOR FIGURES

Fig. 1. Time-dependent loss of apo-nNOS in reaction mixtures containing fraction II. Lanes 1-4, purified apo-nNOS (20 µg/ml) was treated with fraction II, ATP, and ubiquitin as described in 'Experimental Procedures'. Lanes 5-8, as a control the fraction II was boiled for 5 min before addition to the reaction mixture. The apo-nNOS was treated for 0 min (lanes 1 and 5), 30 min (lanes 2 and 6), 60 min (lanes 3 and 7), and 90 min (lanes 4 and 8). The loss of apo-nNOS protein was measured by Western blotting with the use of ¹²⁵I-labeled goat anti-rabbit IgG as the secondary antibody.

Fig. 2. Characterization of the loss of apo-nNOS by fraction II. The loss of apo-nNOS due to fraction II under various conditions was quantified by gamma counting of the band corresponding to nNOS. *A*, indicates the time dependent degradation of nNOS observed in Fig. 1. Closed circles, apo-nNOS with fraction II; open circles, apo-nNOS treated with fraction II after depletion of ATP by prior incubation with 10 units/ml of hexokinase and 10 mM 2-deoxy-D-glucose; closed squares, apo-nNOS treated with boiled fraction II. The values are the mean ± S.E. (n=3). *B*, indicates the effect of MG132 on the loss of apo-nNOS due to fraction II at 90 min. *Inset*, effect of 10 µM calpain inhibitor peptide (*CIP*), 50 µM PD151746 (*PD*), and 1.0 mM EGTA (*EGTA*) on the loss of apo-nNOS due to fraction II after 90 min of incubation. The values are the mean ± S.E. (n=3).

Fig. 3. Effect of ubiquitin-aldehyde on the degradation of the apo-nNOS by fraction II. The degradation of apo-nNOS by fraction II was examined as described for Fig. 2, except that various amounts of ubiquitin-aldehyde were added and 0.2 mg/ml of fraction II was used. The residual amount of apo-nNOS after treatment with fraction II for 90 min is shown. The amount of ubiquitin (Ub) and ubiquitin aldehyde (Ubal) are as indicated. * p < 0.001 vs control. ** p < 0.0004 vs Ub alone.

MOL #000125

Fig. 4. Degradation of the holo-nNOS by fraction II. The degradation of holo-nNOS by fraction II was examined. Holo-nNOS was substituted for apo-nNOS and treated with fraction II as described for Fig. 2. *A*, Western blot of nNOS treated with fraction II in the absence and presence of 10 μ M MG132. Samples were taken at the indicated times, quenched, and later analyzed. *B*, the 125 I-labeled goat anti rabbit antibody bound to nNOS was quantified. Closed squares, holo-nNOS treated with fraction II in the presence of MG132; open squares, holo-nNOS treated with fraction II in the absence of MG132. The values are the mean \pm S.E. (n=3).

Fig. 5. The loss of the SDS-resistant dimeric form of holo-nNOS is independent of proteasomal degradation. The holo-nNOS was treated with fraction II as described and the amount of the SDS-resistant dimeric form (*nNOS dimer*) and the remainder of the nNOS that runs as a monomer (*nNOS monomer*) was measured. As indicated, in some samples 10 μ M MG132 was added to determine if the loss of the dimeric or monomeric nNOS was dependent on proteasomal degradation. *A*, Western blot of the reaction mixture of holo-nNOS treated with fraction II for 0, 30, 60, and 90 min, as indicated. In some samples, MG132 was added as indicated. *B*, the amount of 125 I-labeled goat anti-rabbit IgG was quantified and plotted. The dimeric nNOS are represented by filled symbols and solid lines and the monomeric nNOS is represented by open symbols and dashed lines. Closed squares, dimeric nNOS; closed circles, dimeric nNOS + MG132; open squares, monomeric nNOS; open circles, monomeric nNOS + MG132. The values are the mean \pm S.E. (n=3).

Fig. 6. Effect of 7-nitroindazole (7-NI) and N^G-nitro-L-arginine (NNA) on the amount of SDS-resistant dimer and degradation of nNOS by fraction II. The nNOS was treated with fraction II in the presence of the reversible inhibitor, 7-NI (10 μ M), or the slowly reversible inhibitor, NNA (10 μ M), and the amount of SDS-resistant dimer was measured (panels A and C) as described in Fig. 5. The total amount of nNOS present in these samples was also determined (panels B and D). *A*, the effect of 7-NI on the SDS-resistant dimer. Closed squares, dimeric

MOL #000125

nNOS; closed circles, dimeric nNOS + 7-NI; open squares, monomeric nNOS; open circles, monomeric nNOS + 7-NI. *B*, the effect of 7-NI on the total nNOS protein, which comprises the sum of monomeric and dimeric forms, was quantified as in Fig. 4. Closed circles, nNOS + 7-NI; open squares, nNOS. *C*, the effect of NNA on the SDS-resistant dimer. Closed squares, dimeric nNOS; closed circles, dimeric nNOS + NNA; open squares, monomeric nNOS; open circles, monomeric nNOS + NNA. *D*, the effect of NNA on the total nNOS protein. Closed circles, nNOS + NNA; open squares, nNOS. As a control, heme-deficient apo-nNOS was treated with NNA (closed triangles). The values are the mean \pm S.E. (n=3).

Fig. 7. Heme remains in nNOS during the incubation with fraction II. The nNOS was treated with fraction II in the presence of 10 μ M MG132 and the ferrous-CO complex was measured. Spectrum 1, nNOS mixed with fraction II and immediately processed for analysis; spectrum 2, nNOS incubated with fraction II for 60 min and then processed for analysis; spectrum 3, heme-deficient apo-nNOS. The specific content of the starting holoenzyme in spectrum 1 is 3.6 nmol of P450/mg of protein.

Figure 1

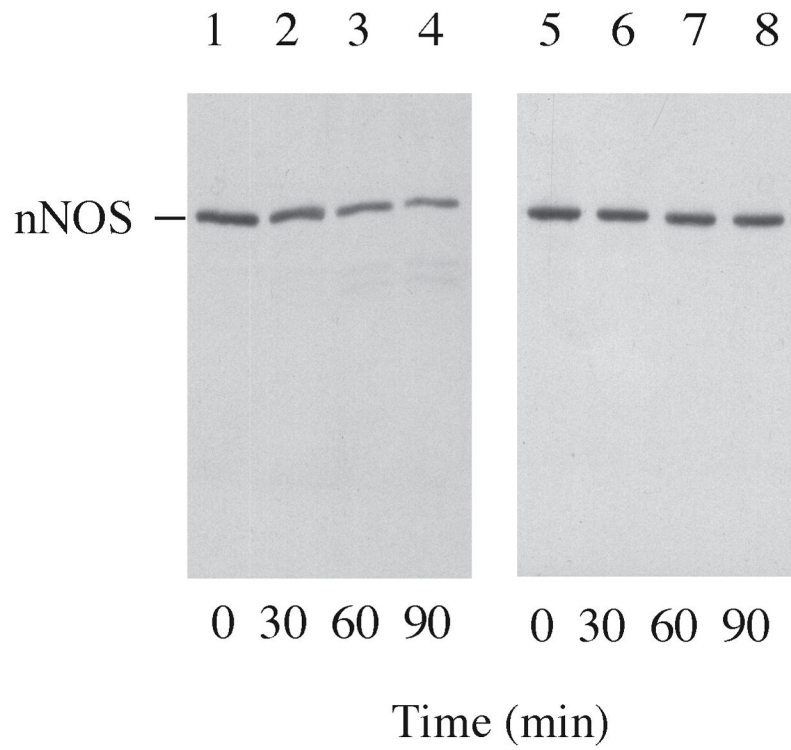


Figure 2

Molecular Pharmacology Fast Forward. Published on July 2, 2004 as DOI: 10.1124/mol.104.000125
This article has not been copyedited and formatted. The final version may differ from this version.

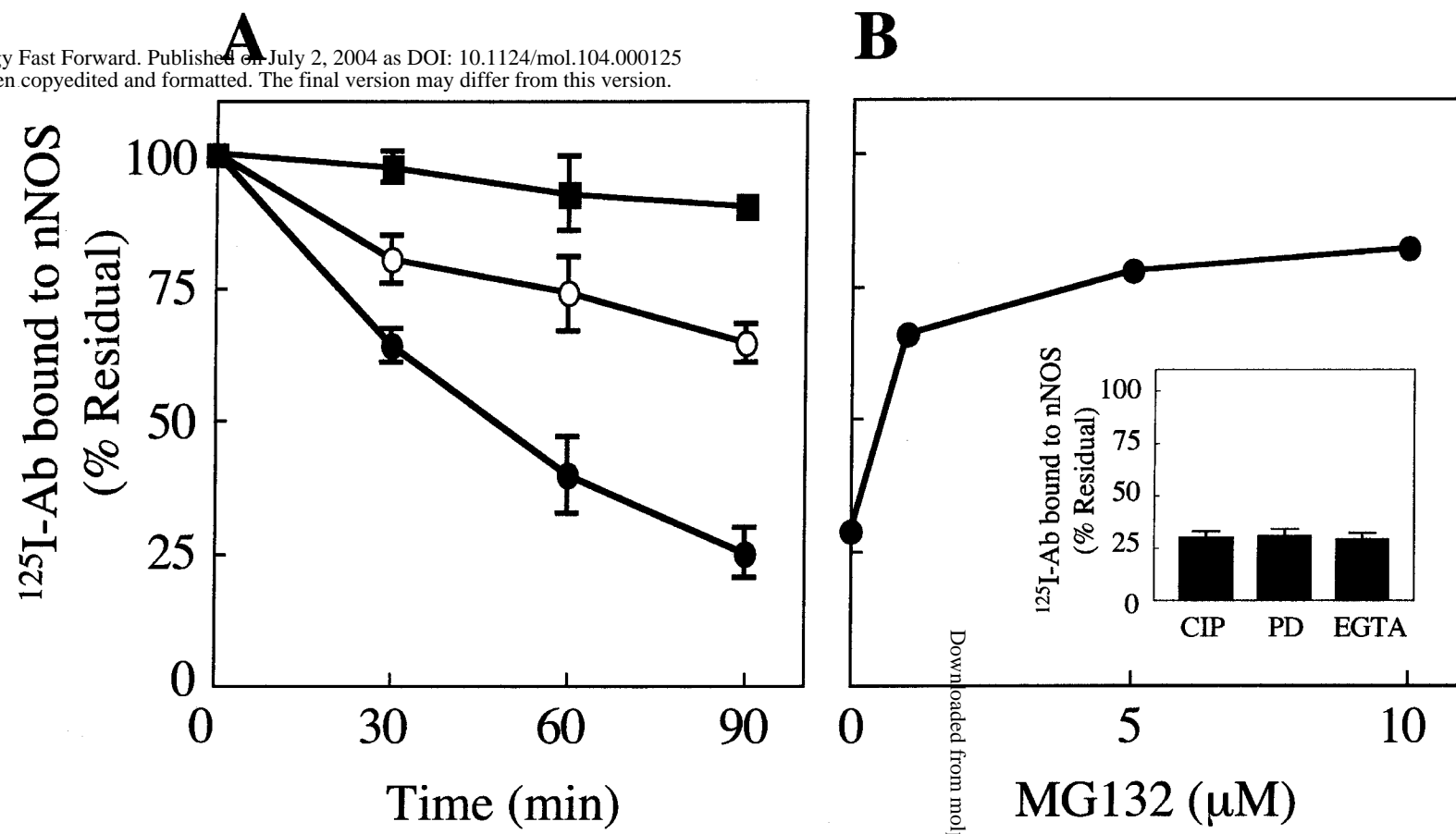
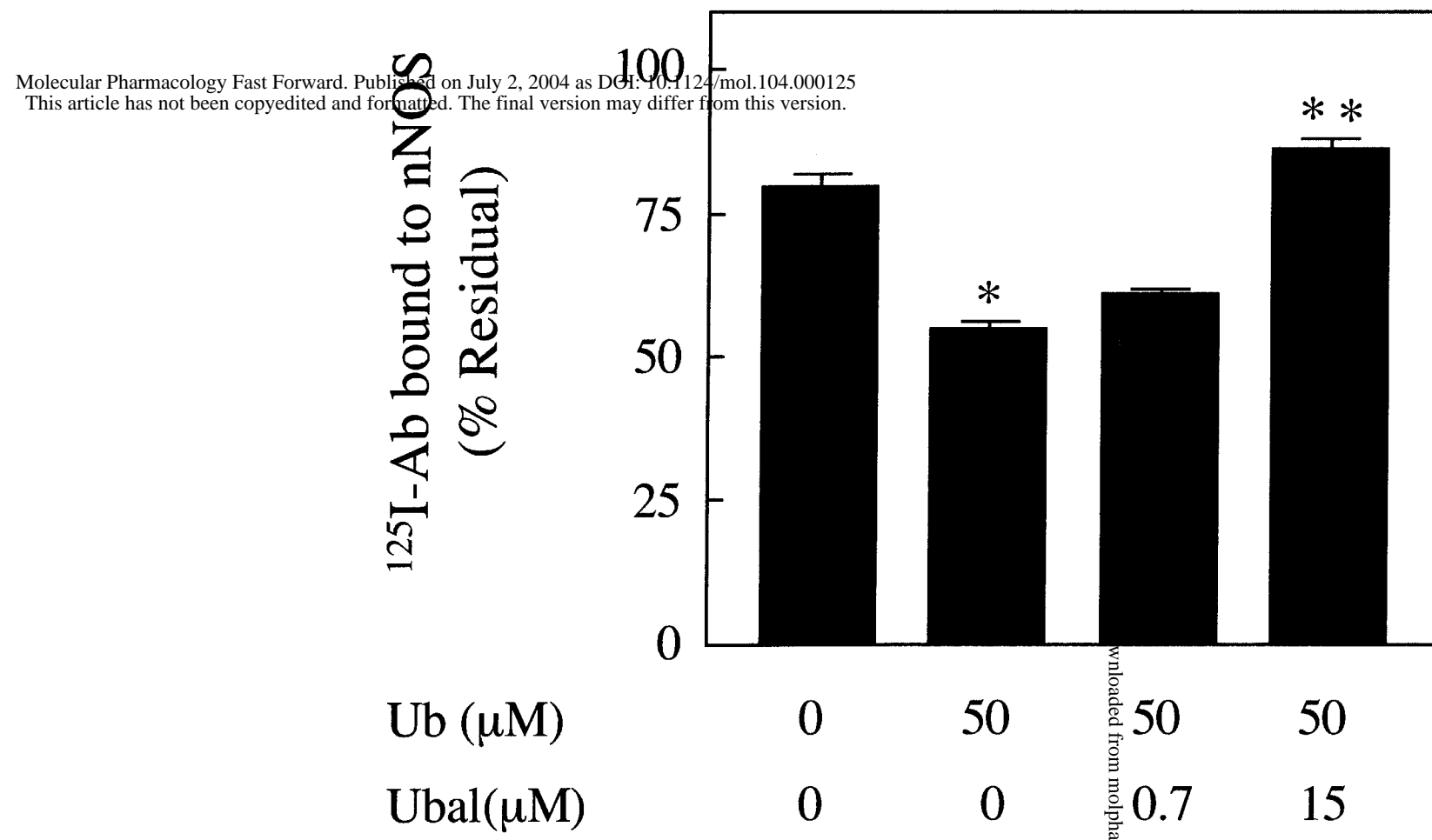


Figure 3



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 4

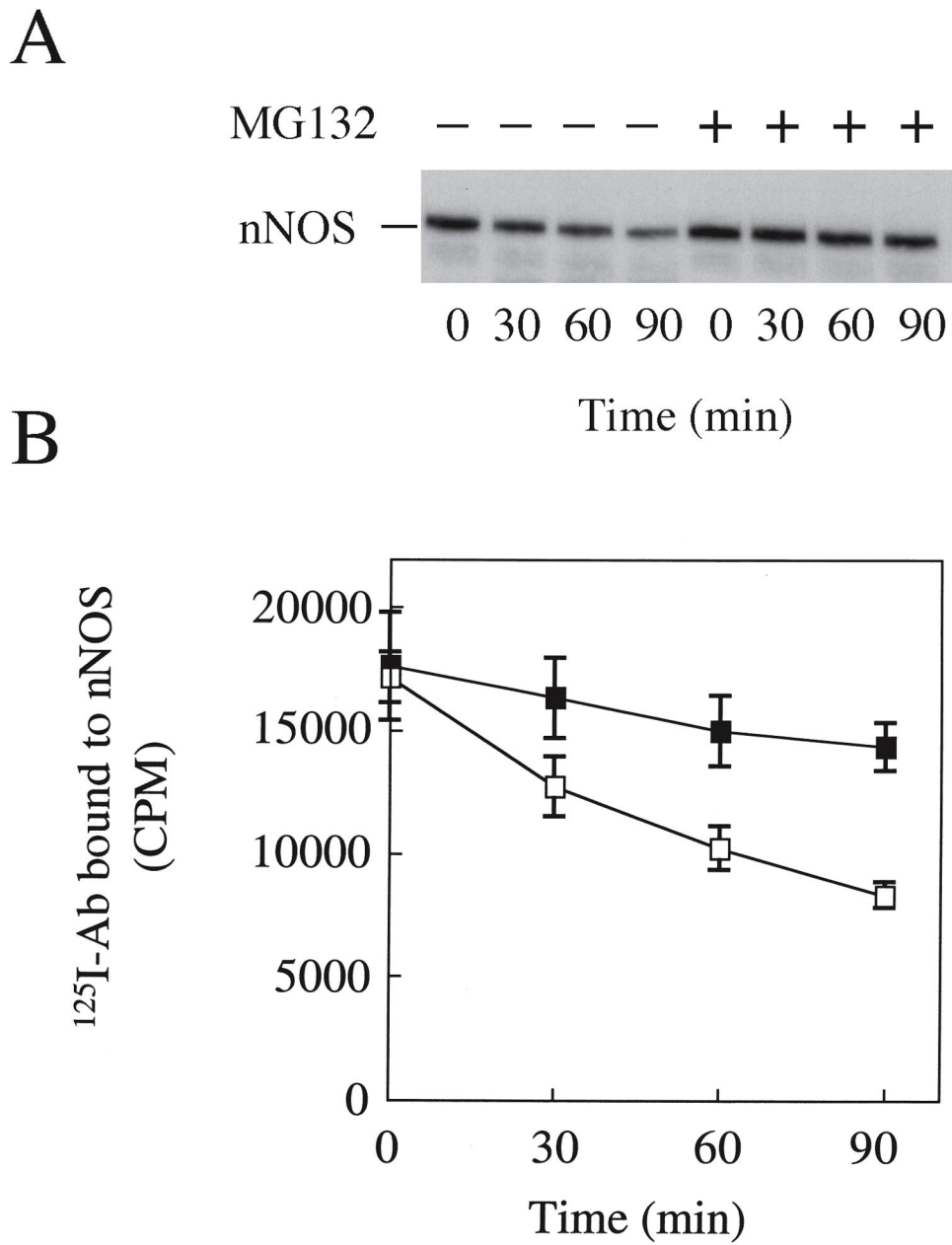


Figure 5

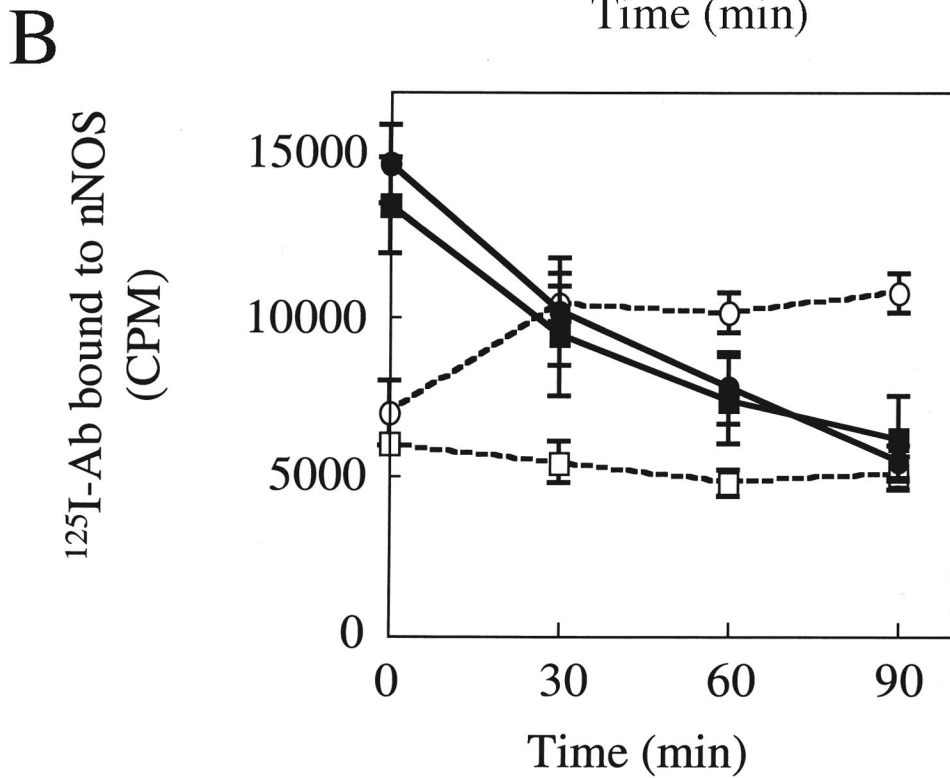
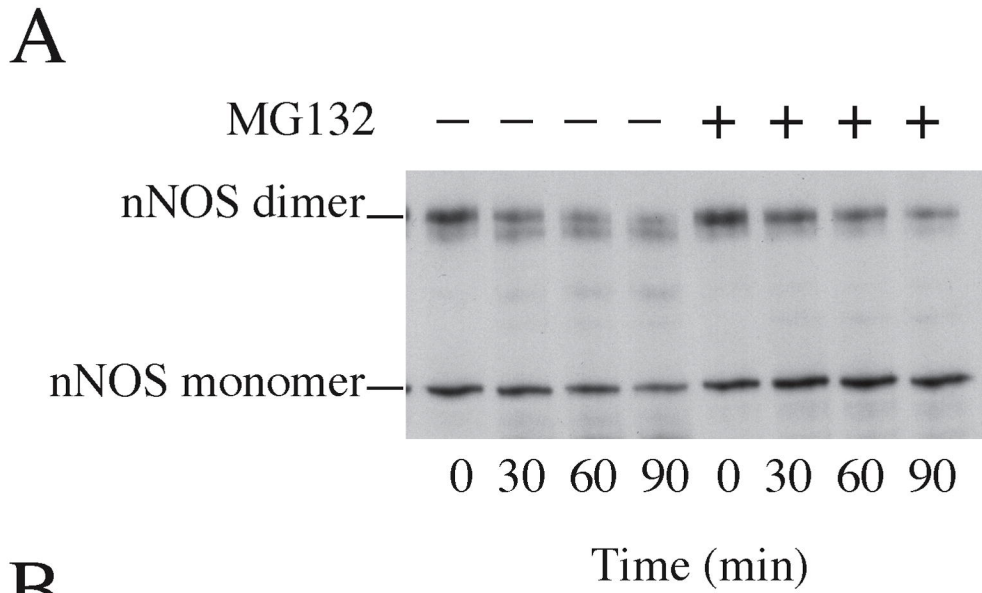
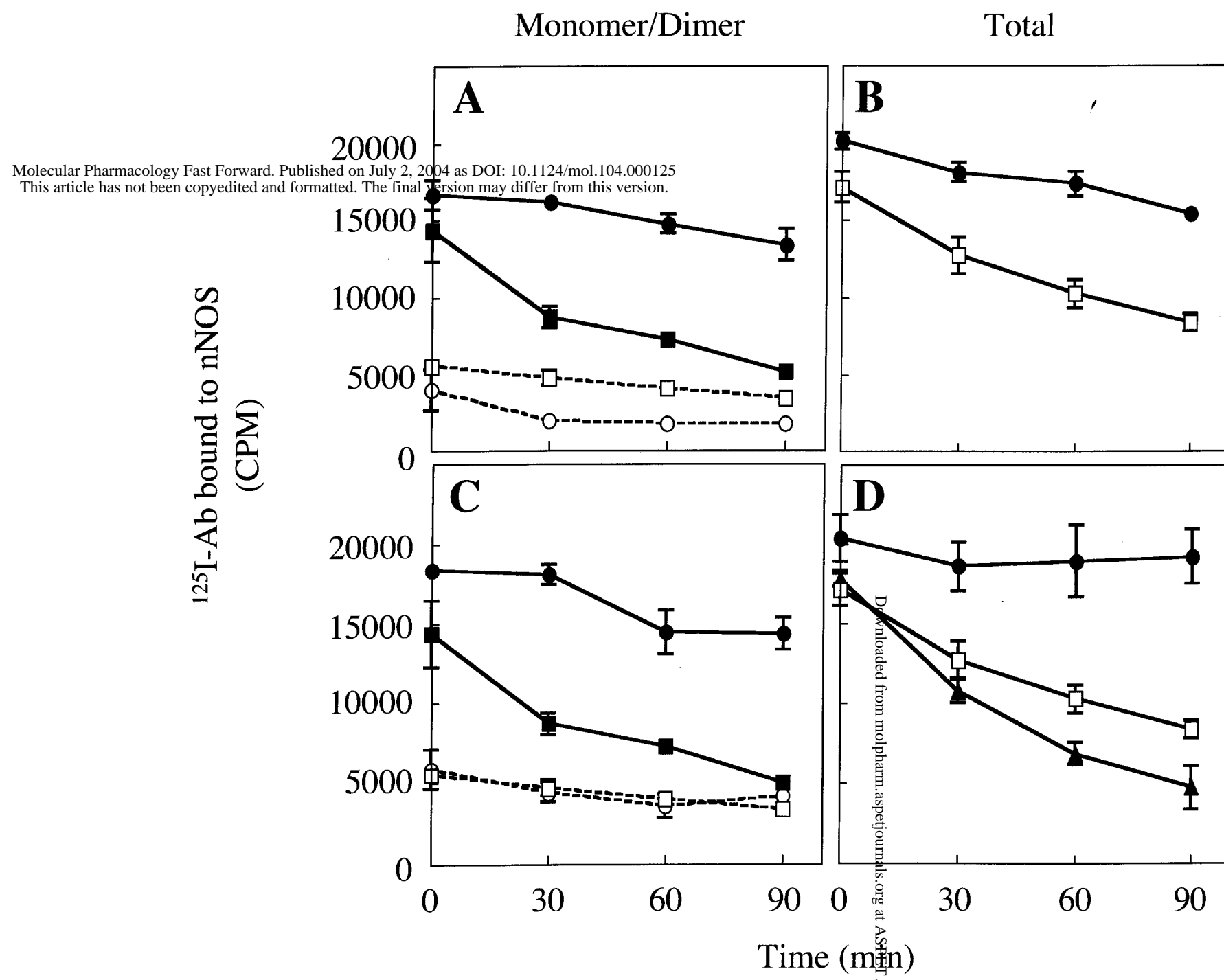


Figure 6



Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 7

