

Ubiquitination and Degradation of Neuronal Nitric-Oxide Synthase in Vitro: Dimer Stabilization Protects the Enzyme from Proteolysis

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ABSTRACT

It is established that neuronal NO synthase (nNOS) is ubiquitinated and proteasomally degraded. The metabolism-based inactivation of nNOS and the inhibition of heat shock protein 90 (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

nNOS (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 seems that the dimeric structure is a major determinant of nNOS stability and proteolysis.

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 (Fellay-Bosco et al., 2000), and NOS inactivators (Noguchi et al., 2000). The effect of inactivators may be of particular impor-

tance in the development of inhibitors for clinical use (Mete and Connolly, 2003).

In the case of the neuronal isoform of NOS (nNOS), metabolism-based inactivators, which act by the 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 (Jianmonkol et al., 2000; Vuletich et al., 2002) and lead to formation

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ABBREVIATIONS: NOS, nitric-oxide synthase; hsp, heat shock protein; nNOS, neuronal nitric-oxide synthase; apo-nNOS, heme-deficient nNOS; holo-nNOS, heme-sufficient nNOS; PAGE, polyacrylamide gel electrophoresis; P450, cytochrome P450; MG132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; BH₄, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; 7-NI, 7-nitroindazole; NNA, N^G -nitro-L-arginine; PD151746, 3-(5-fluoro-3-indolyl)-2-mercapto-(Z)-2-propenoic acid; DE52, diethylaminoethyl cellulose.

of the heme-deficient apo-nNOS. It is interesting to note that 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 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 probably not a prerequisite for degradation and that dimer instability is a molecular trigger for ubiquitination and proteasomal degradation.

Materials and Methods

Materials. (6*R*)-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-Aldrich (St. Louis, MO). The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from BD Biosciences Transduction Laboratories (Lexington, KY). ¹²⁵I-labeled antibody against rabbit IgG was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). MG132 and 7-nitroindazole were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). PD151746 was from Calbiochem (San Diego, CA). Ubiquitin aldehyde was from Alexis Biochemicals (San Diego, CA). Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). DE52 was purchased from Whatman (Climax, NJ).

Expression and Purification of holo-nNOS and apo-nNOS. nNOS was overexpressed in Sf9 insect cells as described previously (Bender et al., 1999). To express holo-nNOS, oxyhemoglobin (25 μM) was added as a source of heme during the last 24 h 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 aprotinin, 6 mM phenylmethylsulfonyl fluoride, and 10 μM BH₄, and the suspended cells were ruptured by Dounce homogenization. Lysates from infected Sf9 cells (8 × 10⁹) were centrifuged at 100,000*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 previously (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 Centrplus YM-10 concentrator (Millipore Corporation, Bedford, MA) to 10 ml and loaded onto a Sephacryl S-300 HR gel filtration column (2.6 × 100 cm; Amersham Biosciences Inc., Piscataway, NJ) 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 μM 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 μM BH₄ and concentrated with the use of a Centrplus YM-10 concentrator. This Sephacryl-purified nNOS preparation had a specific activity of approximately 1000 nmol/min/mg 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 protein.

Treatment of apo-nNOS and holo-nNOS with Fraction II. Fraction II was prepared from rabbit reticulocyte lysates as described previously (Hershko et al., 1983), except that there were two successive DE52 chromatography steps. Each preparation of fraction II was tested with carboxymethylated bovine serum albumin as a positive control. The nNOS preparations (2 μg) were incubated at 37°C in a total volume of 120 μl of 50 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol, and 50 μM ubiquitin; an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, 5 mM MgCl₂, and 10 U/ml creatine phosphokinase); and 2 mg/ml fraction II. At indicated times, the samples were quenched with 25 μl of sample buffer containing 5% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.02% bromphenol blue in 125 mM Tris-HCl, pH 6.8. The samples were boiled for 3 min, and an aliquot (25 μl) was submitted to 6% SDS-PAGE (10 × 8 cm). Proteins were then transferred to nitrocellulose membranes (0.2 μm; Bio-Rad, Hercules, CA) 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 (Eastman Kodak, Rochester, NY) for 1 h at -80°C. The bands corresponding to nNOS were excised, and the radioactivity was quantified by the use of a γ counter. This method was quantitative up to 0.5 μg of nNOS with a linear relationship between the amount of nNOS and radioactivity (*r*² = 0.99).

In studies in which the SDS-resistant dimer was measured, the reaction mixture was quenched with sample buffer supplemented with 100 μM BH₄ and 100 μM L-arginine. The samples were kept on ice before loading for analysis by SDS-PAGE as described above. This method has been described previously by Klatt et al. (1995) to prevent the dissociation of nNOS dimers before and during electrophoresis.

Heme Measurements. The heme present in nNOS was assessed by measuring the ferrous-CO complex. Because 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 × 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 described previously (McMillan et al., 1992). The protein was measured by Coomassie Plus Protein Reagent from Pierce Chemical (Rockford, IL) with the use of bovine serum albumin as standard. The heme content per protein of nNOS was determined to be 0.56 ± 0.06 mol P450/mol monomer of nNOS (mean ± S.D., *n* = 3). The FAD content per protein was measured by high-performance liquid chromatography analysis with fluorescence detection on 2.4 μg of nNOS (Klatt et al., 1996). The assay was linear from 1 to 30 pmol of FAD (*r*² = 0.998). The FAD content is 0.77 ± 0.11 mol/mol monomer of nNOS (mean ± S.D., *n* = 4). Of course, these values are taken from the crude estimation of the protein amounts, and thus the true molar value of the protein is suspect. However, comparison of the heme content with that of the FAD content removes the error caused by the protein measurement and indicates that the heme content is approximately 0.7 mol/mol.

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. Al-

though this may be caused by 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 ^{125}I -labeled secondary antibody (Fig. 2A). There was an approximately 75% loss of apo-nNOS over 90 min (●) compared with less than 10% loss when boiled fraction II was used (■). The omission of ATP decreased the amount of apo-nNOS degraded, indicating an ATP-dependent process (○). As shown in Fig. 2B, MG132, a proteasome inhibitor, protected apo-nNOS from degradation in a concentration-dependent manner. Because the omission of ATP or the 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 and Ortiz de Montellano, 1998); however, as shown in the Fig. 2B inset, calpain inhibitor peptide, PD151746, and EGTA at concentrations that have been reported to inhibit calpain (Laine and Ortiz de Montellano, 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 support ubiquitination ligation reactions that could easily be detected by an antiubiquitin 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 with that observed with 10 μM MG132 under these conditions. The addition of ubiquitin aldehyde at low concentrations (condition 3) is known to inhibit deubiquitinating enzymes (Shaeffer and Cohen, 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 caused by 0.7 μM ubiquitin aldehyde compared with control samples (data not shown), which 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 antiubiquitin antibody, whereas ubiquitin aldehyde at a higher concentration (15 μM) 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 with that performed

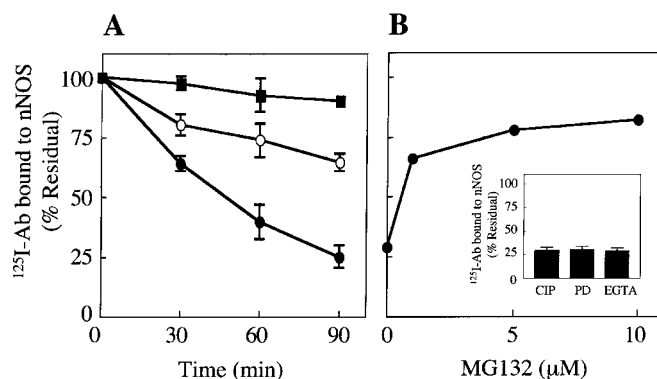


Fig. 2. Characterization of the loss of apo-nNOS by fraction II. The loss of apo-nNOS caused by fraction II under various conditions was quantified by γ -counting of the band corresponding to nNOS. A, the time-dependent degradation of nNOS observed in Fig. 1. ●, apo-nNOS with fraction II; ○, apo-nNOS treated with fraction II after depletion of ATP by prior incubation with 10 U/ml of hexokinase and 10 mM 2-deoxy-D-glucose; ◐, apo-nNOS treated with boiled fraction II. The values are the mean \pm S.E. ($n = 3$). B, the effect of MG132 on the loss of apo-nNOS caused by fraction II at 90 min. Inset, effect of 10 μM calpain inhibitor peptide (CIP), 50 μM PD151746 (PD), and 1.0 mM EGTA on the loss of apo-nNOS caused by fraction II after 90 min of incubation. The values are the mean \pm S.E. ($n = 3$).

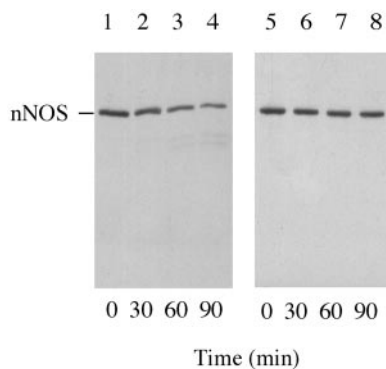


Fig. 1. Time-dependent loss of apo-nNOS in reaction mixtures containing fraction II. Lanes 1 to 4, purified apo-nNOS (20 $\mu\text{g}/\text{ml}$) was treated with fraction II, ATP, and ubiquitin as described under *Materials and Methods*. Lanes 5 to 8, as a control, the fraction II was boiled for 5 min 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 ^{125}I -labeled goat anti-rabbit IgG as the secondary antibody.

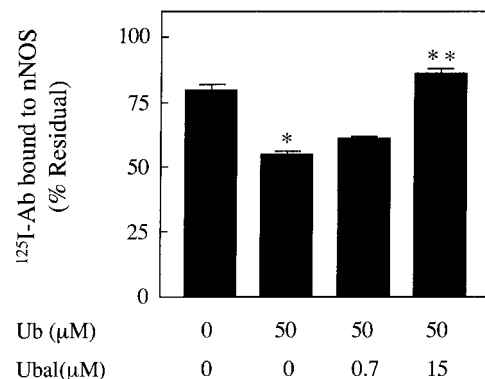


Fig. 3. Effect of ubiquitin-aldehyde on the degradation of the apo-nNOS by fraction II. The degradation of apo-nNOS by fraction II was examined as described for Fig. 2, except that various amounts of ubiquitin-aldehyde were added, and 0.2 mg/ml fraction II was used. The residual amount of apo-nNOS after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

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, □), albeit not as extensively as apo-nNOS above (compare with Fig. 2A, ●). The loss of holo-nNOS was largely attenuated by MG132, indicating that degradation was predominantly by the proteasome (■), 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 (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. The presence of MG132 clearly had no effect on the loss of dimeric nNOS (compare ■ with ●). It is interesting to note that the band corresponding to the mono-

meric form of nNOS accumulated during the incubation period when MG132 was present (compare ○ with □), indicating that the monomeric form is proteasomally degraded. There seems to be little loss of the band corresponding to the monomeric form in the absence of MG132 (□), most likely caused by 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 (compare ■ with ●). This stabilization of the dimer prevented the replenishment of the monomeric pool and resulted in a nearly complete loss of the monomeric form (○). 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 the presence of 10 μ M 7-nitroindazole (compare □ with ●). Highly similar results were obtained for 10 μ M *N*^G-nitro-L-arginine (Fig. 6, C and D). Moreover, *N*^G-nitro-L-arginine had no protective effect on the degradation of apo-nNOS (Fig. 6D, ▲), indicating the specificity of action for the holoenzyme. Thus, it seems that destabilization of the dimeric nNOS, most likely

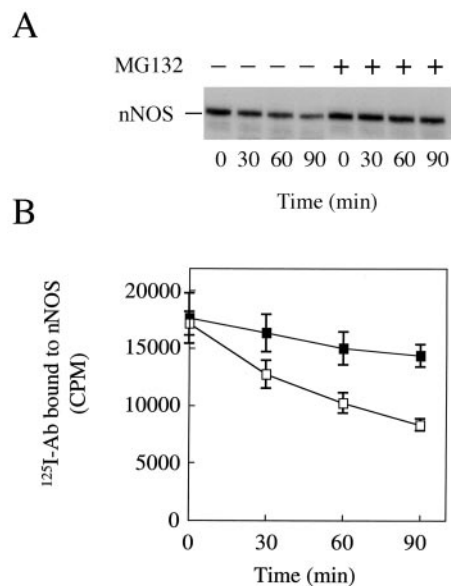


Fig. 4. Degradation of the holo-nNOS by fraction II. The degradation of holo-nNOS by fraction II was examined. Holo-nNOS was substituted for apo-nNOS and treated with fraction II as described for Fig. 2. A, Western blot of nNOS treated with fraction II in the absence and presence of 10 μ M MG132. Samples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

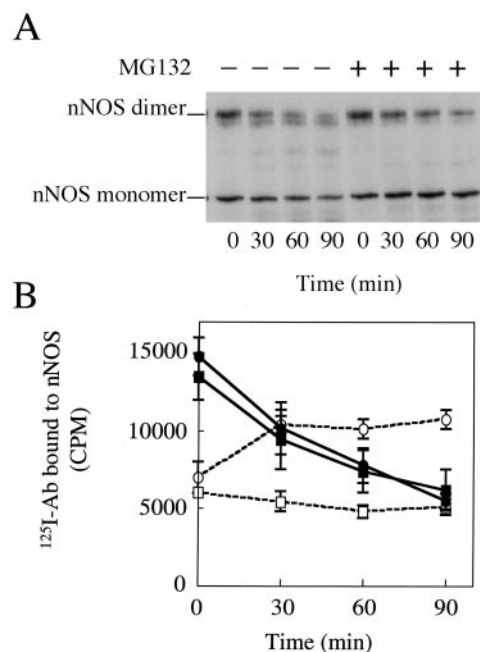


Fig. 5. The loss of the SDS-resistant dimeric form of holo-nNOS is independent of proteasomal degradation. The holo-nNOS was treated with fraction II as described, and the amount of the SDS-resistant dimeric form (nNOS dimer) and the remainder of the nNOS that runs as a monomer (nNOS monomer) were measured. As indicated, in some samples, 10 μ M MG132 was added to determine whether 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 ¹²⁵I-labeled goat anti-rabbit IgG was quantified and plotted. The dimeric nNOS is represented by filled symbols and solid lines, and the monomeric nNOS is represented by open symbols and dashed lines. ■, dimeric nNOS; ●, dimeric nNOS + MG132; □, monomeric nNOS; ○, monomeric nNOS + MG132. The values are the mean \pm S.E. ($n = 3$).

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 μ M MG132 still retains approximately 90% of the original ferrous-CO complex (compare spectrums 1 and 2). Thus, there is very little apo-nNOS formed from holo-nNOS during the incubation with fraction II. From 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 caused by 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.

Discussion

NOS is ubiquitinated and proteasomally degraded *in vivo* (Bender et al., 2000a; Noguchi et al., 2000). In the current study, we used an *in vitro* system to examine the "trigger" for this proteasomal degradation. The *in vitro* system uses 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 (Bender et al., 1999; Noguchi et al., 2000; 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 probably 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 seems 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 associated dimer (Bender et al., 2000b), a heme-containing monomeric form, or heme-deficient apo-nNOS. It seems 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 seems not to be

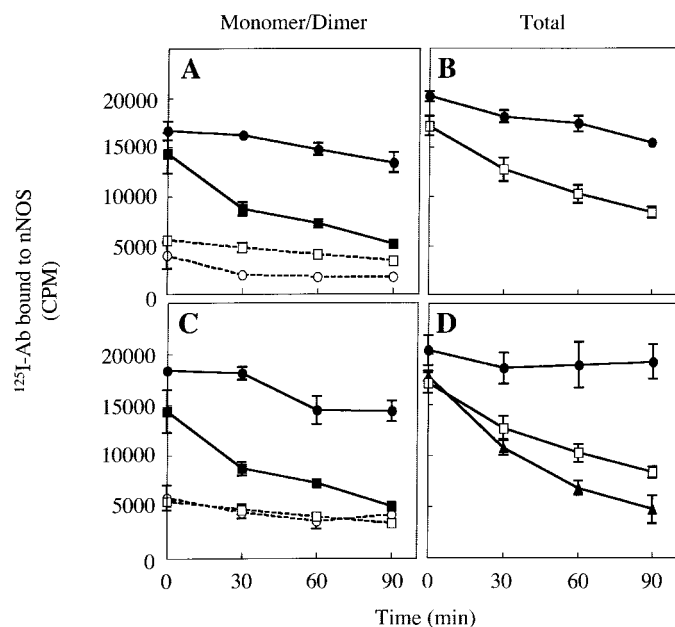


Fig. 6. Effect of 7-NI and NNA on the amount of SDS-resistant dimer and degradation of nNOS by fraction II. The nNOS was treated with fraction II in the presence of the reversible inhibitor 7-NI (10 μ M) or the slowly reversible inhibitor NNA (10 μ M), and the amount of SDS-resistant dimer was measured (A and C) as described in Fig. 5. The total amount of nNOS present in these samples was also determined (B and D). A, the effect of 7-NI on the SDS-resistant dimer. ■, dimeric nNOS; ●, dimeric nNOS + 7-NI; □, monomeric nNOS; ○, 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. ●, nNOS + 7-NI; □, nNOS. C, the effect of NNA on the SDS-resistant dimer. ■, dimeric nNOS; ●, dimeric nNOS + NNA; □, monomeric nNOS; ○, monomeric nNOS + NNA. D, the effect of NNA on the total nNOS protein. ●, nNOS + NNA; □, nNOS. As a control, heme-deficient apo-nNOS was treated with NNA (▲). The values are the mean \pm S.E. ($n = 3$).

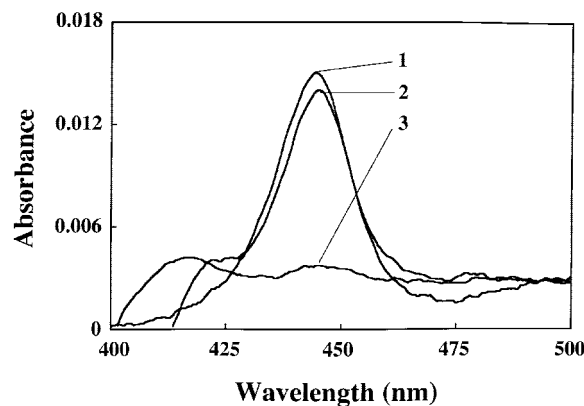


Fig. 7. Heme remains in nNOS during the incubation with fraction II. The nNOS was treated with fraction II in the presence of 10 μ M MG132, and the ferrous-CO complex was measured. Spectrum 1, nNOS mixed with fraction II and immediately processed for analysis. Spectrum 2, nNOS incubated with fraction 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 P450/mg protein.

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 believed to be caused by steric factors that mask the degradation signal. On the other hand, 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 seem to be beneficial.

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