C331A Mutant of Neuronal Nitric-oxide Synthase Is Labilized for Hsp70/CHIP (C Terminus of HSC70-interacting Protein)-dependent Ubiquitination*

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It is established that suicide inactivation of neuronal nitric-oxide synthase (nNOS) by drugs and other xenobiotics leads to ubiquitination and proteasomal degradation of the enzyme. The exact mechanism is not known, although it is widely thought that the covalent alteration of the active site during inactivation triggers the degradation. A mechanism that involves recognition of the altered nNOS by Hsp70 and its cochaperone CHIP, an E3-ubiquitin ligase, has been proposed. To further address how alterations of the active site trigger ubiquitination of nNOS, we examined a C331A nNOS mutant, which was reported to have impaired ability to bind l-arginine and tetrahydrobiopterin. We show here that C331A nNOS is highly susceptible to ubiquitination by a purified system containing ubiquitinating enzymes and chaperones, by the endogenous ubiquitinating system in reticulocyte lysate fraction II, and by intact HEK293 cells. The involvement of the altered heme cleft in regulating ubiquitination is confirmed by the finding that the slowly reversible inhibitor of nNOS, N^G-nitro-l-arginine, but not its inactive D-isomer, protects the C331A nNOS from ubiquitination in all these experimental systems. We also show that both Hsp70 and CHIP play a major role in the ubiquitination of C331A nNOS, although Hsp90 protects from ubiquitination. Thus, these studies further strengthen the link between the mobility of the substrate-binding cleft and chaperone-dependent ubiquitination of nNOS. These results support a general model of chaperone-mediated protein quality control and lead to a novel mechanism for substrate stabilization based on nNOS interaction with the chaperone machinery.

Nitric-oxide synthases (NOS) are cytochrome P450-like hemoprotein enzymes that catalyze the conversion of l-arginine to nitric oxide and citrulline by a process that requires NADPH and molecular oxygen (1). There are three major mammalian isoforms as follows: neuronal NOS (nNOS), 2 endothelial NOS, and inducible NOS. NOS is bidomain in structure with an oxygenase domain, which contains the binding site for the heme, l-arginine, and tetrahydrobiopterin, and a reductase domain, which contains the binding sites for FMN, FAD, and NADPH (2). NOS is a highly regulated enzyme requiring homodimerization and bound calmodulin for efficient electron transfer from the flavins to the heme moiety to enable synthesis of NO. Another mechanism of regulation is the ubiquitination and proteasomal degradation of NOS (3). Of particular pharmacological interest is the finding that certain drugs cause the suicide inactivation, covalent alteration, ubiquitination, and proteasomal degradation of nNOS (3–8). This phenomenon is not unique to nNOS as it is well documented that the suicide inactivation of other P450 cytochromes leads to covalent alteration, enhanced ubiquitination, and proteasomal turnover of the enzymes (9). The C terminus of Hsc70-interacting protein (CHIP) has been shown to be an E3 ligase that ubiquitinates cytochromes P450 3A4 and 2E1 as well as nNOS (10–12). The ubiquitination of nNOS by CHIP is enhanced in the presence of Hsp70 (8, 10). Moreover, suicide-inactivated nNOS was found to be selectively ubiquitinated by this Hsp70/CHIP system (8). Other E3 ligases, such as gp78 for CYP3A4 (12) and Parkin for nNOS (13), have been identified and point to a redundancy in ubiquitin ligases for P450 cytochromes. Nonetheless, the Hsp70/CHIP-mediated ubiquitination of inactivated NOS is a valuable model for the study of the cellular machinery that culls altered P450 enzymes.

The mechanism of how these inactivated P450 enzymes, including inactivated nNOS, are culled for ubiquitination and degradation is not known, although it is widely thought that the covalent alteration of the active site during suicide inactivation of the enzyme somehow triggers the ubiquitination. The covalent alterations that have been characterized include modification of the heme prosthetic group, modification of amino acid residues near the active site, as well as cross-linking of the heme to the protein (9, 14, 15). In the case of nNOS, we have found that covalent alteration of the enzyme-bound tetrahydrobiopterin also plays a role in labilizing the enzyme for ubiquitination and degradation (4, 16). At present, we do not know whether a slight opening of the active site cleft is a sufficient trigger for nNOS ubiquitination or if a more global unfolding of the enzyme must occur. Suicide inactivators covalently alter specific active site moieties, and this argues for a slight alteration of the heme substrate-binding cleft as the trigger; however, global unfolding cannot be ruled out. This is especially true in cases where the heme prosthetic group is destroyed, and the apoprotein is formed (15).
In the course of studies on the ubiquitination of nNOS, we have recently found that a C331A mutant of nNOS is highly susceptible to ubiquitination. This particular residue is one of two cysteine residues in a CXXXC motif that provide the thiolate ligation for a tetradeutate zinc-binding site (17). The zinc is a structural and not a catalytic feature of nNOS. The C331A nNOS mutant was first described by Masters and co-workers (18) and found to affect tetrahydrobiopterin and L-arginine binding. As first isolated, this mutant was found to be inactive and unable to bind tetrahydrobiopterin, although the heme prosthetic moiety was intact. Interestingly, prolonged incubation of C331A nNOS with high concentrations of L-arginine restores the ability to bind tetrahydrobiopterin and synthesizes NO (18). In this study we characterize the ubiquitination of this active form of C331A nNOS as a genetic model for a labilized form of nNOS. We show that C331A nNOS is labilized for ubiquitination by a purified system of ubiquitinating enzymes and chaperones, by the endogenous ubiquitinating system in reticulocyte lysate fraction II, and by intact HEK293 cells. Moreover, the C331A nNOS is stabilized by N⁶-nitro-L-arginine, a slowly reversible inhibitor that is known to stabilize wild type nNOS from ubiquitination and degradation. The D-isomer had no effect, indicating that the effects of N⁶-nitro-L-arginine are specific for the heme active site cleft of C331A nNOS. Thus, certain ligands are able to stabilize a conformation of C331A nNOS that is resistant to ubiquitination. We have also verified that C331A nNOS is ubiquitinated in an Hsp70/CHIP-dependent manner and that Hsp90 opposes the ubiquitination, as was described previously for wild type nNOS (8, 10). Thus, the pathways for ubiquitination are the same for wild type and mutant enzymes. These studies indicate the importance of conformational changes involving the heme active site cleft in determining nNOS interaction with Hsp70 that triggers ubiquitination of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). (6R)-5,6,7,8-Tetrahydro-L-1-biopirterin (BH₄) was purchased from the Schirck laboratory (Jona, Switzerland). Protein A-Sepharose, ubiquitin, ATP, creatine phosphokinase, L-arginine, N⁶-nitro-L-arginine, N⁶-nitro-D-arginine, A23187, and rabbit polyclonal anti-nNOS were purchased from Sigma. HRP-tagged goat anti-rabbit secondary antibody was from Chemicon (Temecula, CA). Rabbit polyclonal anti-CHIP antibody and N-acetyl-Leu-Leu-norleucinal was from Calbiochem. MG132 was purchased from Biomol (Plymouth Meeting, PA). GST-tagged ubiquitin, ubiquitin aldehyde, and ubiquitin-activating enzyme (E1) were from Boston Biochem (Cambridge, MA). Creatine phosphatase was from Fluka (St. Louis, MO). Complete Mini protease inhibitor mixture was from Roche Diagnostics. Nickel-nitrilotriacetic acid-agarose was from Qiagen Inc. (Valencia, CA). The cDNA for rat neuronal nNOS was kindly provided by Dr. Solomon Snyder (The Johns Hopkins Medical School, Baltimore). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (University of Michigan). The cDNA for expressing the UbcH5a-GST fusion protein was kindly provided by C. M. Pickart (The Johns Hopkins Medical School). pET30a CHIP plasmid for expressing His-CHIP was kindly provided by C. Patterson (University of North Carolina, Chapel Hill).

**Methods**

**Expression and Purification of nNOS, C331A nNOS, Hsp70, Hsp40, Hsp90, GST-tagged UbcH5a, and CHIP**—The C331A construct in pCW was made using the site-specific mutagenesis approach described by Martasek et al. (18). The C331A-nNOS and wild type nNOS pCW plasmid was transfected as described previously (19), except that BL21 *Escherichia coli* cells were used. The bacterially overexpressed wild type and mutant nNOS was purified by 2',5'-ADP-Sepharose and Mono Q ion exchange chromatography as described previously (20), except that 10 μM BH₄ was present in the buffers used during purification. The enzyme as isolated was active, and further incubation with L-arginine did not increase the activity. His-CHIP was bacterially expressed and purified by nickel-nitrilotriacetic acid affinity chromatography as described previously (21). Hsp90 and Hsp70 were purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as described previously (22). YDJ-1, the yeast ortholog of Hsp40, was expressed in bacteria and purified by sequential chromatography on DE52 and hydroxylapatite as described previously (22). GST-tagged UbcH5a (E2, ubiquitin carrier protein) was bacterially expressed and purified by GSH-Sepharose affinity chromatography as described previously (23).

**Assay for NOS Activity**—NO synthesis activity was determined by measuring the conversion of oxyhemoglobin to methemoglobin. Aliquots (5.3 μl) of the reconstitution mixtures were added to an assay mixture containing 100 μM CaCl₂, 100 μM L-arginine, 100 μM BH₄, 100 units/ml catalase, 10 μg/ml calmodulin, 25 μM oxyhemoglobin, and an NADPH-regenerating system consisting of 400 μM NADP⁺, 10 mM glucose 6-phosphate, and 1 unit/ml glucose-6-phosphate dehydrogenase, expressed as final concentrations, in a total volume of 180 μl of 50 mM potassium phosphate, pH 7.4. The mixture was incubated at 37 °C, and the rate of oxidation of oxyhemoglobin was monitored by measuring the absorbance at λ₄01–411 nm with a microtiter plate reader.

**In Vitro Ubiquitination of nNOS by DE52-retained Fraction of Reticulocyte Lysate**—The DE52-retained fraction of rabbit reticulocyte lysate (fraction II) was prepared as described previously (24). Purified nNOS (0.2 μM) was incubated for 1 h at 37 °C with 4.5 μl of DE52-retained fraction (final concentration 7 mg of protein/ml), 0.3 mg/ml bovine serum albumin, 10 μM BH₄, 8.3 μM GST-tagged ubiquitin, 1 mM dithiothreitol, 10 mM ATP/Mg²⁺, 1 μl of Complete Mini protease inhibitor mixture, 0.6 mM N-acetyl-Leu-Leu-norleucinal, and 0.8 μM ubiquitin aldehyde (deubiquitination inhibitor), adjusted to a final volume of 20 μl with 50 mM Tris, pH 7.5. Incubations were terminated by boiling with an equal volume of SDS-sample buffer containing 8 M urea and 2 M thiourea. In some studies, an Hsp90-free pool of fraction II eluted from DE52 with a gradient of KCl was prepared and denoted as fraction A (25). Fraction A (final concentration 5.5 mg/ml), which contained Hsp70,
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**A**

| kDa | Short exposure | Long exposure |
|-----|----------------|---------------|
| 150 |                |               |
| 100 |                |               |
| 50  |                |               |
| 25  |                |               |
| 10  |                |               |
| GST-Ub | - | - | + | + |
| nNOS-poly-Ub | - | - | + | - |
| nNOS-mono-Ub | - | - | + | - |
| nNOS | - | - | - | - |

**B**

![Image](image-url)

**C**

![Image](image-url)

**FIGURE 1.** C331A nNOS mutant is preferentially ubiquitinated in fraction II by a process that is attenuated by N^2^-nitro-L-arginine but not N^2^-nitro-D-arginine. The ubiquitination of purified wild type and C331A mutant of nNOS by fraction II was performed as described under “Experimental Procedures.” A, nNOS-ubiquitin conjugates produced by fraction II without (lane 1) and with (lane 2) GST-ubiquitin. The blot depicted in the right panel is the same blot as the left panel except that the blot was exposed to film for longer time to obtain the signals corresponding to the polyubiquitin bands. B, time-dependent formation of a higher molecular mass band that corresponds to the mono-ubiquitinated nNOS (nNOS-mono-Ub). A representative blot is shown in the upper panel. The nNOS-mono-Ub was quantified by the use of ImageJ software (National Institutes of Health) and plotted in the lower panel. C331A nNOS ubiquitin conjugates; squares, wild type nNOS ubiquitin conjugates. The values are the mean ± S.E. (n = 3). C, effect of N^2^-nitro-L-arginine (L-NNA) or N^2^-nitro-D-arginine (D-NNA) on the ubiquitination of C331A mutant of nNOS (C331A). The N^2^-nitro-arginine compound was added to the ubiquitination mixture to give a final concentration of 25 μM, and the amount of nNOS-mono-Ub formed after 60 min of incubation was determined. The ubiquitinated nNOS was measured as in B, and the values are the mean ± S.E. (n = 3). Statistical probability is expressed as follows: ***, p < 0.001.

Hsp40, and nNOS ubiquitination activity, replaced fraction II in some ubiquitination assays.

**In Vitro Ubiquitination of nNOS by Purified Ubiquitinating System**—The nNOS was pretreated with Hsp70 and Hsp40 and then ubiquitinated. In these experiments, 5.0 μM Hsp70 and 0.5 μM Hsp40 were incubated for 20 min at 30 °C with 1.5 μM nNOS, 10 μM BH₄, and 2.5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase) in a total volume of 25 μl of 50 mM Hapes, pH 7.5. The reaction mixture was then added to nNOS, an aliquot (5 μl) of this reaction mixture was incubated with a purified system containing an E1 ubiquitin-activating enzyme (0.1 μM), an E2 GST-tagged UbcH5a (1.5 μM), His-tagged CHIP (4.0 μM), GST-tagged ubiquitin (8.3 μM), 1 mM DTT, 10 mM MgCl₂, and 10 mM ATP, expressed as final concentrations, for 1 h at 30 °C in a total volume of 20 μl of 50 mM Tris-Cl, pH 7.5. After incubation, 20 μl of sample buffer was added, and an aliquot (22 μl) was loaded for Western blotting.

Cell Culture and Transient Transfection—Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s minimum essential medium supplemented with 10% (v/v) bovine calf serum. Transient transfections of HEK293T cells were carried out with the use of a standard calcium phosphate method as described previously (26) in 10-cm plates. The wild type and C331A mutant of rat nNOS cDNA was subcloned from PVL1393 (27) into the EcoRI and NotI sites of pcDNA3.1 (+). His-HA-Ub cDNA in pcDNA3 was obtained from Dr. Yi Sun (University of Michigan). His-HA-Ub cDNA (4 μg) and nNOS cDNA (3.5 μg) were transfected into 70–80% confluent cells such that the total amount of cDNA was kept constant with vector plasmid. Cells were transfected for 48 h and, where indicated, were treated with MG132 (10 μM) for the indicated times prior to harvesting. SDS-PAGE, Western Blotting, and Immunoprecipitation—Cells from two 10-cm plates were harvested at 48 h after transfection and combined, washed with ice-cold phosphate-buffered saline (PBS), and sonicated in 0.4 ml of HS buffer (10 mM Hapes, pH 7.4, 0.32 M sucrose, 2 mM EDTA, 6 mM PMSE, 10 mg/ml leupeptin, 2 mg/ml aprotinin, 10 mg/ml trypsin inhibitor, 10 mM sodium vanadate, 1% (w/v) Nonidet P-40, and 5 mM N-ethylmaleimide). Homogenates were centrifuged for 30 min at 14,000 × g, and the supernatant was taken for immunoprecipitation of nNOS. The nNOS was immunoabsorbed from ~3 mg of HEK293 cytosol with 15 μl of anti-nNOS IgG and 70 μl of protein A-Sepharose (20%, w/v slurry) in a total volume of 400 μl of HS buffer for 2 h at 4 °C. Immune pellets were added to 40 μl of sample buffer containing 5% (w/v) SDS, 20% (v/v) glycerol, 6 mg/ml DTT, and 0.02% (w/v) bromphenol blue in 125 mM Tris-HCl, pH 6.8. After boiling, 40 μl of the samples were resolved on 6% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes for 2 h at 100 V. The blot was probed with anti-nNOS (0.01%, w/v), and an HRP-tagged goat anti-rabbit secondary antibody (0.0025%, w/v) and the immunoreactive bands were then visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) and X-Omat film (Eastman Kodak Co.). The film was scanned, and the
nNOS-Ub was quantified by the use of ImageJ software (National Institutes of Health).

Nitrite and Nitrate Assay—HEK293T cells cultured in 10-cm plates were treated with A23187 (10 μM) for 2 h in 10 ml of medium. Aliquots of medium (200 μl) were taken for assay of nitrite and nitrate as described previously (28). In this procedure, nitrate reductase (Roche Diagnostics) was used to convert the nitrate to nitrite, which was quantified by the use of the Griess reagent. Sodium nitrate in culture medium was used as a standard.

RESULTS

C331A nNOS Is Preferentially Ubiquitinated by Fraction II—With the use of a DE52-retained fraction of reticulocyte lysate (fraction II), we compared the ubiquitination of the purified C331A mutant of nNOS with that of the wild type enzyme. We have previously used the fraction II system, which has been extensively used to study protein ubiquitination (24), to characterize the ubiquitination of wild type nNOS (8). In this system, the predominant ubiquitin conjugate detected is the mono-ubiquitinated form, although polyubiquitin conjugates also form. The monoubiquitin conjugate is the predominant species in human embryonic kidney cells and rat brain cytosol (6). We used GST-tagged ubiquitin so that the conjugates could be more easily detected by Western blotting with anti-nNOS IgG. As shown in Fig. 1A, the major ubiquitin conjugate of the C331A nNOS is also the monoubiquitinated form (lane 2, left panel), although polyubiquitin conjugates, which can be visualized with extended exposure time, also form (lane 2, right panel). As will be shown later, the changes in monoubiquitin levels parallel those of the polyubiquitin levels. For simplicity, we chose to focus on quantification of the monoubiquitin conjugate in our studies. Moreover, quantification of the monoubiquitin conjugate of the wild type nNOS has already shown that both functional inactivation of nNOS with suicide inactivators and inactivation of Hsp90 enhance nNOS ubiquitination (8). This is similar to observations made in intact cells and in vivo (7, 27). As shown in Fig. 1B, the time-dependent increase in the ubiquitination of C331A nNOS (triangles) is much greater than that of the wild type enzyme (squares). The ubiquitination is sensitive to ligands of nNOS. As shown in Fig. 1C, the L-, but not D-, isomer of N⁶-nitroarginine decreases the ubiquitination of C331A nNOS.

As shown in Fig. 2A, the omission of tetrahydrobiopterin from the incubation mixture has no effect on the ubiquitination of the C331A nNOS (open bars, cf. 1st with 2nd condition) but greatly increases the ubiquitination of the wild type nNOS (solid bars, cf. 3rd with 4th condition). The labilized wild type nNOS is protected from ubiquitination by L-, but not D-, N⁶-nitroarginine (Fig. 2A, solid bars, cf. 5th with 6th condition). In Fig. 2B, the concentration dependence of N⁶-nitro-L-arginine for protection against ubiquitination is presented for both the wild type (squares) and the C331A mutant of nNOS (triangles). The N⁶-nitro-L-arginine protects the wild type nNOS and the C331A nNOS with an IC₅₀ of 0.2 ± 0.3 and 8.7 ± 0.3 μM, respectively. The greater than 40-fold difference in IC₅₀ values for N⁶-nitro-L-arginine likely reflects the greater conformational flexibility of the heme/substrate cleft inherent in C331A nNOS over that of the pterin-deficient wild type nNOS.

Role of Hsp70 and CHIP in the Ubiquitination of C331A nNOS by Fraction II—CHIP, an Hsp70 cochaperone, has been shown to be an E3 ligase for nNOS by overexpression studies in HEK293T cells and in in vitro studies with purified CHIP and Hsp70 (10). We examined the role of Hsp70 and CHIP in fraction II on the ubiquitination of C331A nNOS. Methylene blue has been shown to inhibit the ATPase activity of Hsp70 (29) and to be a useful reagent for defining Hsp70-dependent ubiquitination (30). As shown in Fig. 3A, methylene blue decreases the fraction II-mediated ubiquitination of C331A nNOS in a concentration-dependant manner (lanes 2–6). Approximately 85% inhibition of ubiquitination is observed at 1 μM methylene blue (Fig. 3A, lane 5), and this inhibition is substantially blunted by the addition of purified Hsp70 (Fig. 3A, lane 7). Thus, C331A nNOS ubiquitination is dependent on Hsp70. As indicated earlier, the changes measured in the monoubiquitin conjugate...
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FIGURE 3. Hsp70 and CHIP dependence of the ubiquitination of C331A nNOS catalyzed by fraction II. The ubiquitination of C331A nNOS catalyzed by fraction II was performed as described under “Experimental Procedures,” except that the indicated concentrations of methylene blue (MB) were added. Samples were Western-blotted by probing with anti-nNOS and an HRP-tagged goat anti-rabbit secondary antibody. A, relative amounts of nNOS-Ub from replicate experiments were determined by scanning, expressed as relative density, and plotted. Lane 1, time 0; lanes 2–7, time 60. The values are the mean ± S.E. (n = 3). B, amount of mono-ubiquitin conjugate in each sample in A was plotted against the amount of polyubiquitin conjugates. The relative amount of polyubiquitin conjugate was determined by scanning the area depicted in Fig. 1A. A line was fit to the data with an r² of 0.84. C, CHIP is the major ubiquitin ligase for C331A nNOS ubiquitination. The C331A nNOS was incubated with fraction II as above except that 2% (w/v) nonimmune serum or 1% (v/v) or 2% (v/v) anti CHIP serum was added as indicated. Lane 1, incubation time 0; lanes 2–4, incubation time 1 h. Statistical probability is expressed as follows: **, p < 0.01; ***, p < 0.001.

reflect the changes seen in the polyubiquitin conjugates. As shown in Fig. 3B, both the monoubiquitin and polyubiquitin conjugates of C331A nNOS were quantified in samples from studies in Fig. 3A and plotted. There is a linear correlation (r² = 0.84) between the mono- and polyubiquitin conjugates formed.

As shown in Fig. 3C, the addition of 1% anti-CHIP antibody to the fraction II ubiquitination mixture decreases the ubiquitination of C331A nNOS by 40% (lane 4). At a concentration of 2% anti-CHIP serum, nearly complete inhibition of ubiquitination by reticulocyte lysate fraction II is observed (Fig. 3C, lane 5). This suggests that CHIP is the major ubiquitin ligase for C331A nNOS in fraction II. As a control, we found that 2% nonimmune serum has no significant effect on the ubiquitination (Fig. 3C, lane 3).

Ubiquitination of C331A nNOS in an in vitro System Containing Purified Ub Ligases—The ubiquitin ligase activity of CHIP has been observed in an in vitro system containing other ubiquitin ligase for C331A nNOS. The omission of added Hsp70 (condition 7) decreased ubiquitination by ~40%, consistent with previous reports showing that the purified preparations of nNOS have Hsp70 as a contaminant (10).

Stability of C331A nNOS and Wild Type nNOS and the Effect of Arginine on the Stability and Ubiquitination of C331A nNOS in Vitro—We wished to further define the nature of the C331A nNOS that is targeted for ubiquitination. The purified C331A had a K_m and V_max of 11.1 ± 1.8 μM and 443 ± 74 nmol/min/mg protein, respectively, whereas the wild type enzyme purified in the same manner gave a K_m and V_max of 8.8 ± 1.6 μM and 424 ± 46 nmol/min/mg protein, respectively. Thus, the active form of C331A nNOS was similar to that of the wild type enzyme. The C331A nNOS is known to be unstable in the absence of L-arginine (18), and we wondered if this inactive form is the actual substrate for ubiquitination. Thus, we wished to compare the stability of the C331A nNOS to that of the wild type nNOS and required components of a ligase system, including purified E1 and E2 (31–33). CHIP was shown to be a ubiquitin ligase for nNOS with the use of an in vitro system containing purified nNOS, E1, E2, Hsp70, Hsp40, and CHIP (10). We have used the same system to determine whether CHIP acts as an ubiquitin ligase for C331A nNOS. As shown in Fig. 4A, the purified ubiquitinating system in the presence of GST-tagged ubiquitin gives rise to predominantly polyubiquitin adducts of C331A nNOS consistent with that previously described for wild type nNOS (10). Under the current conditions of excess tetrahydrobiopterin, there was greater ubiquitination of C331A nNOS over that for the wild type nNOS. The polyubiquitin conjugates that were detected in the area indicated by the brace were quantified and plotted in Fig. 4B. The C331A nNOS is ubiquitinated to a greater degree than the wild type enzyme (Fig. 4B, cf. condition 1 with condition 2). Moreover, the L-, but not D-, N⁶-nitroarginine protects C331A nNOS from ubiquitination by the purified protein system (Fig. 4B, cf. condition 3 with condition 4). Thus, the findings with purified proteins are highly similar to those obtained with fraction II. As shown in Fig. 4B, the omission of CHIP (condition 6) decreases the ubiquitination of C331A nNOS to the level seen in the absence of GST-ubiquitin (condition 5). Thus, CHIP functions as a

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FIGURE 5. C331A nNOS is destabilized and becomes a substrate for ubiquitination. A, catalytic stability of wild type nNOS and C331A nNOS was examined. Purified C331A nNOS (2 μl) was incubated at 30 °C with 10 μM BH4 in a total volume of 30 μl of 250 mM Tris, pH 7.5. In some cases, 100 μM L-arginine or D-arginine was added to incubation mixtures containing C331A nNOS. At the indicated times, an aliquot (5.3 μl) was taken to assess nNOS activity by the oxyhemoglobin method described under “Experimental Procedures.” Open squares, wild type nNOS; solid squares, C331A nNOS; solid circles, C331A nNOS with 100 μM L-arginine; solid triangles, C331A nNOS with 100 μM D-arginine. B, effect of L-arginine (L-arg) and D-arginine (D-arg) on the ubiquitination of C331A nNOS in the in vitro system containing purified E1, E2, and CHIP, as in Fig. 4. Statistical probability is expressed as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

the effect of L-arginine on stability and ubiquitination. As shown in Fig. 5A, the C331A nNOS (solid squares) is completely inactivated over a period of 90 min, which is the duration of time for the ubiquitination assay above. Over the same time, the wild type nNOS (Fig. 5A, open squares) maintains approximately half of its activity. When 100 μM L-arginine is present, C331A nNOS is stabilized to the level of wild type enzyme (Fig. 5A, solid circles). D-Arginine at the same concentration does not stabilize the C331A nNOS (Fig. 5A, solid triangles). As shown in Fig. 5B, the ubiquitination of C331A nNOS is reduced to approximately one-half in the presence of L-arginine. The ubiquitination of C331A nNOS with L-arginine is approximately the level of ubiquitination found for the wild type nNOS in Fig. 4B. The D-arginine did not affect the ubiquitination of C331A nNOS (Fig. 5B). Thus, the inactive form of C331A nNOS, which was initially described by the Masters and co-workers (18), appears to be the substrate for ubiquitination.

Effect of Hsp90 on the Ubiquitination of C331A nNOS—We recently reported that Hsp90 opposes CHIP-mediated ubiquitination of wild type nNOS in vitro (8). We wished to determine whether Hsp90 similarly inhibits the ubiquitination of C331A nNOS. To examine this, we prepared an Hsp90-free pool consisting of reticulocyte lysate proteins eluting from DE52 prior to Hsp90, as described previously (25). The fraction pool that elutes before Hsp90 contains Hsp70, Hsp40, and Hsp70, and we call it fraction A (25). As described earlier, the C331A nNOS is unstable, and the enzyme activity decreases over time. As shown in Fig. 6A, fraction A ubiquitinates C331A nNOS, and the addition of purified ubiquitin (GST-Ub, lane 5), CHIP (lane 6), or Hsp70 (lane 7) are also shown. Statistical probability is expressed as follows: **, p < 0.01; ***, p < 0.001.

As described earlier, the C331A nNOS is unstable, and the enzyme activity decreases over time. As shown in Fig. 6B, the addition of Hsp90 to C331A nNOS at a concentration that nearly completely blocks ubiquitination does not protect against the activity loss even over a 15-min time period. Thus, we see protection against C331A nNOS ubiquitination but not against the loss of activity.
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A

![Graph A](attachment:imagea.png)

nNOS-mono-Ub

nNOS

Hsp90 (µM) 0 3.3 6.6 13.2

**nNOS-mono-Ub (relative intensity)**

B

![Graph B](attachment:imageb.png)

nNOS Activity (mmol NO/minute/mg)

Time (min) 0 15 15 + 15

Hsp90

***

* Time (min) 0 15 15 + 15

**DISCUSSION**

We have previously shown that suicide inactivation of wild type nNOS leads to enhanced ubiquitination and proteasomal degradation of the enzyme by a process that involves CHIP and Hsp70 (6–8, 27). CHIP is an E3 ubiquitin ligase that binds through its TPR domain to a TPR acceptor site on Hsp70 and mediates the ubiquitination of a variety of Hsp90-chaperoned proteins (21, 35), including that of nNOS (8). Although we now know that CHIP binds to nNOS-bound Hsp70 to direct ubiquitination of the nNOS, we do not yet understand how the suicide-inactivated nNOS is selected for ubiquitination. Suicide inactivators covalently alter important active site moieties and lead to irreversible inactivation of the enzyme. We envision that these covalent modifications lead to opening of the nNOS substrate-binding cleft as the initial stage of unfolding that is recognized by Hsp70, thus facilitating CHIP-directed ubiquitination of the altered nNOS (36). Although much has been learned by the use of suicide inactivators in labilization of wild type nNOS, the complexity of the alterations and the instability of the adducts are major limitations in the study of these processes.

Studies by Masters and co-workers (18) showed that a mutation of cysteine residue 331 of nNOS to an alanine perturbs the ω-arginine and tetrahydrobiopterin-binding cleft of nNOS, and we wondered if such a conformational change would be sufficient for triggering ubiquitination. In this study, we have shown that the C331A mutation destabilizes nNOS and renders the protein more susceptible to ubiquitination in in vitro systems containing endogenous or purified ubiquitinating enzymes and chaperones, as well as in an intact HEK293T cell model. We also show in the in vitro systems that ubiquitination of C331A nNOS is Hsp70- and CHIP-dependent. Thus, we suggest here that the C331A mutant of nNOS functions as a model for the “labilized” state of nNOS, a state similar to that achieved by suicide inactivation of the wild type enzyme. The cysteine residue at position 331 serves as a binding site for zinc at the nNOS dimer interface, and at first glance, its mutation would appear not to have an effect on the active site cleft but rather to play a critical role in dimer stability. The C331A nNOS can exist in an inactive form, which does not bind tetrahydrobiopterin or ω-arginine very well (18). This inactive form of C331A nNOS can be reversibly activated upon incubation in the presence of...
The importance of the substrate-binding cleft in regulating ubiquitination is also apparent from studies with N-nitroarginine. We show here that N⁵-nitro-L-arginine, which is a slowly reversible inhibitor of nNOS that binds at the heme active site, protects the tetrahydrobiopterin-deficient wild type nNOS and C331A nNOS from ubiquitination. Previously, it has been established that the loss of tetrahydrobiopterin labilizes the wild type nNOS for ubiquitination in vitro, in intact cells, and in vivo (4, 5, 16). The inhibition must occur through interaction at the active site, as the biologically inactive stereoisomer N⁵-nitro-D-arginine does not show any protection of either wild type nNOS or C331A nNOS. Thus, the substrate-binding cleft controls the ubiquitination of C331A nNOS, consistent with the notion that suicide inactivation and alteration of the binding cleft trigger ubiquitination and degradation.

We speculate that perturbation of the heme cleft through mutation or covalent modification by suicide inactivators leads to a more flexible or dynamic state of nNOS heme cleft that is more open and progresses to a partially unfolded state of the enzyme (36). This conformational change allows Hsp70 to interact with hydrophobic elements of the cleft interior as they are exposed during the opening/unfolding process. Although it is clear from our studies that heme cleft perturbations play a role in the labilization of nNOS, we do not understand precisely what stage of the cleft opening process is recognized by Hsp70. Because dimer stability appears to be intimately related to substrate cleft conformation, we cannot be certain if the actual recognition site is on the cleft, at the dimer interface, or at another site (5, 37–39). The importance of the cleft in regulating ubiquitination is also consistent with the enhanced ubiquitination seen for guanabenz-inactivated nNOS, which is tetrahydrobiopterin-deficient due to the oxidative destruction of the pterin during suicide inactivation (4). In this case, the suicide inactivation appears highly specific for tetrahydrobiopterin as no heme or protein adducts are detected (4). Consistent with the critical role of tetrahydrobiopterin in regulating turnover, it has been shown that a decrease in tetrahydrobiopterin level (without suicide inactivation) is itself sufficient to enhance the ubiquitination and proteasomal degradation of nNOS (5, 16). Tetrahydrobiopterin binds in the active

of high concentrations of L-arginine (18). We have found that the presence of tetrahydrobiopterin during the isolation process maintains the C331A nNOS in the active state, and this is the preparation of enzyme that we used in the this study. This active form of C331A nNOS had Kₘ and Vₘₐₓ values for L-arginine similar to that of the wild type nNOS. The expression of C331A nNOS in HEK293T cells catalyzes NO formation after treatment with the calcium ionophore to a level near that found for the wild type enzyme. Thus, the C331A nNOS appears to have a fully functional active site in cells. In the absence of high concentrations of L-arginine or tetrahydrobiopterin, the C331A nNOS is unstable, and this inactive form is likely the form that is ubiquitinated. This is apparent from our stability studies in vitro where we correlated the stability of C331A nNOS to the ubiquitination. In particular, L-arginine, but not D-arginine, stabilized the C331A nNOS and protected the enzyme from ubiquitination. Thus, stability of the nNOS is regulated by the state of the active site heme cleft.

FIGURE 7. C331A nNOS is preferentially ubiquitinated in HEK293T cells. The ubiquitination of nNOS in HEK293T cells was determined after transient co-transfection with ubiquitin and immunoprecipitation as described under “Experimental Procedures.” A, immunoblot showing the ubiquitin conjugate of wild type nNOS (WT) or C331A mutant of nNOS. The time indicates the duration of time the cells were treated with MG132. B, ubiquitin conjugate of wild type (square) or C331A mutant (triangle) of nNOS that was formed in the HEK293T cells was quantified and plotted. C, effect of the treatment of cells with N⁵-nitro-L-arginine (L-NNA) or N⁵-nitro-D-arginine (D-NNA) on the ubiquitination of wild type (solid bars) or the C331A mutant of nNOS (open bars). Cells were pretreated for 1 h with 0.4 mM N⁵-nitro-arginine and then treated with MG132 for 2 h. D, nitrate and nitrite produced from wild type expressing cells (solid bars) and C331A nNOS expressing cells (open bars). Cells were treated for 1 h with 4 μM calcium ionophore (A23187) alone or with 0.4 mM N⁵-nitro-L-arginine. The amount of nitrate and nitrite released into the medium was assayed by the Griess method as described under “Experimental Procedures.” The amounts of wild type and C331A nNOS in the HEK293T cells were comparable, as determined by Western blotting. The values given in all panels are the mean ± S.E. (n = 3). Statistical probability is expressed as follows: *, p < 0.05; **, p < 0.001.
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site in intimate association with the heme and participates in catalysis. Because exogenous tetrahydrobiopterin can readily replenish pterin-deficient nNOS and protect it from ubiquitination, it appears that only a slight perturbation of the active site conformation is sufficient to trigger Hsp70/CHIP-dependent ubiquitination and degradation of nNOS (4, 5, 16).

One potential model for the more flexible or dynamic state of the heme cleft is the slight opening of the heme cleft of myoglobin found after suicide inactivation by bromotrichloromethane (40), where a reactive intermediate covalently modifies the heme prosthetic group to a protein-bound product (41). Cross-linking of heme to protein has been shown to be an important signal for cytochrome P450 hemoprotein degradation (15, 42). The structure of the heme-myoglobin adduct was determined by two-dimensional NMR, and subsequent molecular dynamics simulation of the altered myoglobin showed that the heme active site was more open allowing the access of solvent water molecules to the heme (40, 41). The enhanced oxidase activity found associated with the altered myoglobin is consistent with the increased flexibility of the heme cleft and the access of water molecules (40). Thus, we suggest that perturbations like that found with the heme cleft of myoglobin may be the actual trigger for ubiquitination and degradation.

We have recently proposed a model of nNOS triage that involves opposing actions of Hsp90 and Hsp70, with Hsp90 inhibiting and Hsp70 promoting CHIP-mediated ubiquitination (8, 36). nNOS exists in native complexes with Hsp90, and Hsp90 inhibitors enhance the ubiquitination and turnover of nNOS (3, 6, 27). Hsp90 regulates signaling proteins by modulating ligand-binding clefts (43), and we have suggested that Hsp90 stabilizes a more open state of the nNOS heme/substrate-binding cleft to enhance enzyme activity (36). As we have noted (43), the extent to which a ligand-binding cleft is open determines ligand access and thus protein function, but clefts are inherent sites of conformational instability. When the cleft is opened, whether by chemical alteration or genetic mutation, to such an extent that Hsp90 cannot stabilize the cleft, then Hsp70-dependent ubiquitination can proceed. Indeed, we have shown with the use of purified proteins that Hsp90 opposes the action of Hsp70/CHIP on suicide-inactivated nNOS (8, 36), and as we have shown here, purified Hsp90 protects against ubiquitination of C331A nNOS by reticulocyte lysate proteins.

Hydrophobic clefts are important structural elements in all proteins and are often responsible for the biological activity of the protein. A chaperone-mediated surveillance of cleft perturbations is likely an important process in maintaining cellular protein quality. We are impressed by the profound stabilization of C331A nNOS activity that is provided by the substrate L-arginine and the accompanying decrease in CHIP-dependent ubiquitination illustrated in Fig. 5. This manifestation of the classic phenomenon of substrate stabilization may reflect the ability of the substrate to promote a more closed state of the heme/substrate-binding cleft that is less amenable to interaction with Hsp70 and accordingly CHIP. The notion that substrate stabilization of nNOS may reflect an interaction of the substrate-binding cleft with the chaperones involved in protein quality control may be applicable to substrate stabilization of enzymes in general. This indeed would be a novel mechanistic explanation for a phenomenon that has been known to occur in intact cells for decades.

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