Ubiquitination of Neuronal Nitric-oxide Synthase in the Calmodulin-binding Site Triggers Proteasomal Degradation of the Protein*

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Background: CHIP-dependent ubiquitination of NO synthase is an important regulatory mechanism.

Results: A dozen ubiquitination sites on neuronal NO synthase were identified with 11 of the sites on either the oxygenase or calmodulin domain.

Conclusion: Lysine residue 739 is the major site for poly-ubiquitination with other sites responsible for mono-ubiquitination of neuronal NO synthase.

Significance: CHIP-dependent regulation of neuronal NO synthase turnover occurs primarily through lysine residue 739.

Nitric-oxide synthase, a cytochrome P450-like hemoprotein enzyme, catalyzes the synthesis of nitric oxide, a critical signaling molecule in a variety of physiological processes. Our laboratory has discovered that certain drugs suicide-inactivate neuronal nitric-oxide synthase (nNOS) and lead to the preferential ubiquitination of the inactivated nNOS by an Hsp70- and CHIP (C terminus of Hsc70-interacting protein)-dependent process. To further understand the process by which altered nNOS is recognized, ubiquitinated, and proteasomally degraded, we examined the sites of ubiquitination on nNOS. We utilized an in vitro ubiquitination system containing purified E1, E2 (UbcH5a), Hsp70, and CHIP that recapitulates the ability of the cells to selectively recognize and ubiquitinate the altered forms of nNOS. LC-MS/MS analysis of the tryptic peptides obtained from the in vitro ubiquitinated nNOS identified 12 ubiquitination sites. Nine of the sites were within the oxygenase domain and two were in the calmodulin-binding site, which links the oxygenase and reductase domains, and one site was in the reductase domain. Mutational analysis of the lysines in the calmodulin-binding site revealed that Lys-739 is a major site for polyubiquitination of nNOS in vitro and regulates, in large part, the CHIP-dependent degradation of nNOS in HEK293 cells, as well as in in vitro studies with fraction II. Elucidating the exact site of ubiquitination is an important step in understanding how chaperones recognize and trigger degradation of nNOS.

Nitric-oxide synthase (NOS)² is a cytochrome P450-like hemoprotein enzyme that catalyzes the conversion of L-arginine to L-citrulline and nitric oxide, an important cellular signaling molecule (1). NOS is a homodimer with each monomer having an oxygenase domain, which binds heme, tetrahydrobiopterin, and L-arginine, and a reductase domain, which binds FMN, FAD, and NADPH (2). A calmodulin-binding domain links the oxygenase and reductase domains, and the binding of calmodulin regulates the flow of electrons from the reductase to the oxygenase domain (2). Although a variety of regulatory mechanisms exist for the control of NOS, an important mechanism for the maintenance of NOS protein quality control is through ubiquitination by the C terminus of Hsc70-interacting protein (CHIP), an E3 ubiquitin ligase that binds both Hsp70 and Hsp90 through tetratricopeptide repeat domains (3, 4). CHIP has been shown to be in native complexes with all three NOS isoforms as follows: neuronal NOS (nNOS), endothelial NOS, and inducible NOS (5–8). Although CHIP is not the only ubiquitin ligase for NOS, as Parkin was found to ubiquitinate nNOS (9) and SPSB2 was found to direct the ubiquitination of inducible NOS independent of CHIP (10), the CHIP-dependent pathway appears to be the major one involved in the recognition and ubiquitination of altered dysfunctional forms of NOS (11, 12). The discovery that dysfunctional forms of NOS were selectively culled came from in vitro and in vivo studies on the suicide inactivation of nNOS (13–16). The suicide inactivation caused the covalent alteration of the heme or tetrahydrobiopterin of nNOS, and this has led to the concept that alteration of the structure of the active site cleft of nNOS triggers ubiquitination and degradation of the enzyme (5, 17). Our laboratory has shown that the inactivated nNOS is preferentially ubiquitinated in an in vitro system containing purified Hsp70 and CHIP (6). More recently, greater levels of Hsp70 and CHIP were found associated to nNOS when ubiquitination was enhanced (18). Interestingly, N⁴-nitro-L-arginine, but not the d-isomer, protects against NOS ubiquitination and decreases the levels of Hsp70 and CHIP found in complex with nNOS (18). Moreover, in studies where the individual domains of nNOS were expressed in

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The abbreviations used are: NOS, nitric-oxide synthase; CHIP, C terminus of Hsc70-interacting protein; nNOS, neuronal nitric-oxide synthase; Ub, ubiquitin; NNA, N⁴-nitroarginine; KOR, ubiquitin with all lysines mutated to arginines.

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HEK293T cells, Hsp90 and Hsp70 were found only in complexes with the oxygenase domain (18).

To further understand how the Hsp70/CHIP recognize and triage dysfunctional nNOS, in this study we examined where CHIP ubiquitinates nNOS. To our knowledge, the sites of nNOS ubiquitination are not known. We have identified a dozen sites on nNOS that are ubiquitylated by CHIP with the use of LC-MS/MS methods, and all but one of these sites was found on the oxygenase-caldomin domain of nNOS. We show that lysine residues within the calmodulin-binding domain are important in the CHIP-mediated poly-ubiquitination of nNOS, and this site regulates degradation of nNOS in HEK293 cells. Lysine residue 739 was found to be the predominant site responsible for the ubiquitination and degradation of nNOS. These studies further support the notion that the Hsp70/CHIP interaction with the oxygenase domain is important in maintaining nNOS protein quality control.

EXPERIMENTAL PROCEDURES

Materials—(6R)-5,6,7,8-Tetrahydro-1-biopterin was purchased from the Schirck laboratory (Jona, Switzerland). Protein A-Sepharose, ubiquitin, ATP, creatine phosphokinase, 1-arginine, N^2-nitro-1-arginine, N^2-nitro-d-arginine, and rabbit polyclonal anti-nNOS IgG were purchased from Sigma. Goat anti-rabbit IgG IRDye was purchased from Licor Biosciences. Clonal anti-nNOS IgG were purchased from Sigma. Goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology. Goat anti-Hsp70, Hsp40, GST-tagged UbcH5a, DE52 was purchased from Whatman. Recombinant human Hsp70, Hsp90 and Hsp40, GST-tagged Hsp70, Hsp40, GST-tagged UbcH5a, and GST-Hsp40 were incubated for 30 min at 30 °C with 4 μM nNOS in a total volume of 20 μl of 50 mM Hepes, pH 7.4, containing 100 mM KCl, and 5 mM dithiothreitol (DTT). To conjugate ubiquitin to nNOS, an aliquot (1 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 (10 μM), His-tagged CHIP (3 μM), His-tagged ubiquitin (500 μM), 1 mM DTT, 5 mM ATP in an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase), expressed as final concentrations, for 1 h (unless otherwise indicated) at 30 °C in a total volume of 40 μl of 50 mM Hepes, pH 7.4, containing 100 mM KCl, and 5 mM dithiothreitol (DTT). To conjugate ubiquitin to nNOS, an aliquot (1 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 (10 μM), His-tagged CHIP (3 μM), His-tagged ubiquitin (500 μM), 1 mM DTT, 5 mM ATP in an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase), expressed as final concentrations, for 1 h (unless otherwise indicated) at 30 °C in a total volume of 40 μl of 50 mM Hepes, pH 7.4, containing 100 mM KCl, and 5 mM DTT. After incubation, 80 μl of sample buffer (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) was added, and an aliquot (14 μl) was loaded for Western blotting.

SDS-PAGE and Western Blotting—After boiling, samples were resolved on 5% (w/v) SDS-polyacrylamide gels and transferred to Immobilon FL membranes (Millipore, Billerica, MA) for 2 h at 100 V. The blot was probed with anti-nNOS (0.01%, w/v) and then a goat anti-rabbit IRDye secondary antibody (0.0067%, w/v), and the immunoreactive bands were visual-
ized with the use of the LI-COR Odyssey Imaging System, and the bands were quantified using Image Studio software (LI-COR). The linearity of the detection system was validated with nNOS and ubiquitinated nNOS standards (0.03–0.75 μg of nNOS/lane).

**Purification of nNOS-Ubiquitin Conjugates Using Nickel Magnetic Beads**—For large scale preparation and purification of nNOS-ubiquitin conjugates, incubations were the same as above except that the concentration of nNOS was increased 5-fold to 20 μM in a total volume of 800 μl of the ubiquitination reaction mixture. After the 1-h incubation at 30 °C, the sample was concentrated to 40 μl of equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, pH 8.0) and then added to 400 μl of nickel magnetic beads equilibrated with the same equilibration buffer. The sample was rotated with the beads for 30 min at 4 °C. The supernatant was then removed, and the sample was washed three times with 1 ml of wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, pH 8.0) and then added to 400 μl of nickel magnetic beads equilibrated with the same equilibration buffer. The sample was rotated with the beads for 30 min at 4 °C. The supernatant was then removed, and the sample was washed three times with 1 ml of wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, pH 8.0). After removal of the final wash, 70 μl of SDS-sample buffer was added to the beads. The sample was then boiled, and 65-μl aliquots were then loaded onto 5% SDS-polyacrylamide gels and then stained with Coomassie Blue dye. The appropriate nNOS-ubiquitin conjugate band (molecular mass range of 165–175 kDa) was excised from the gel and sent to MS Bioworks (Ann Arbor, MI) for analysis.

**Identification of nNOS Ubiquitination Sites**—Gel samples were washed with 25 mM ammonium bicarbonate followed by acetonitrile. Samples were then reduced with 10 mM DTT at 60 °C followed by alkylation with 50 mM iodoacetamide at room temperature. Samples were then digested with trypsin (Promega) at 37 °C for 4 h. Following digestion, samples were quenched with formic acid, and the supernatant was analyzed directly by nano-LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Orbitrap Velos Pro mass spectrometer. Peptides were loaded on a trapping column and eluted over a 75-μm analytical column at 350 nl/min; both columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The 15 most abundant ions were selected for MS/MS. The MS/MS data were searched against the SwissProt database using Mascot search engine version 2.4 (Matrix Science, London, UK; version Mascot). Mascot was set up to search the IPI.RAT version 3.75 decoy database assuming the digestion enzyme trypsin with a maximum of two missed cleavages. The precursor and product ion tolerances were 10 ppm and 0.5 Da, respectively. Cyclization of N-terminal glutamine, deamidation of asparagine and glutamine, oxidation of methionine, acetylation of the N terminus, and ubiquitination (Gly-Gly) of lysine residues were specified in Mascot as variable modifications. Carbamidomethylation of cysteines was set as a fixed modification. Mascot DAT files were parsed into Scaffold (Proteome Software, Portland, OR) for visualization and validation using the Prophet tools (22, 23). Minimum protein probability was 90%, and peptide probability was 50%; a minimum of two unique peptides was necessary for protein identification. Putative Gly-Gly-modified peptides were confirmed by manual inspection of the raw MS/MS spectra.

**In Vitro Degradation of nNOS by Fraction II**—DE52-retained fraction II was prepared from rabbit reticulocyte lysate as described previously (24). In studies where nNOS degradation was measured, purified nNOS (2 μg) was incubated at 37 °C in a total volume of 120 μl of 50 mM Tris-HCl pH 7.4, containing 2 mM DTT, 500 μM His-ubiquitin, an ATP-regenerating system, and 2 mg/ml of fraction II. At indicated times, a 25-μl aliquot of each sample was taken and quenched with 25 μl of sample buffer. Samples were then submitted to SDS-PAGE and 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 (25) in 6-well plates. The WT, 7R, 6R739K, and 6R743K rat nNOS cDNA was subcloned from PV1.393 (19) into the EcoRI and NotI sites of pcDNA3.1+. Human CHIP cDNA was in pcDNA3 (8). The four types of nNOS cDNA (0.5 μg) were cotransfected with CHIP cDNA (0, 0.1, 0.5, and 1 μg) 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 lactacystin (10 μM) for 20 h prior to harvesting. To harvest the cells, the media were removed, and cells were gently washed once with PBS. After removing the wash, 250 μl of sample buffer was added to the wells, and the cells were agitated with a pipette tip. Samples were then boiled for 5 min and then subjected to SDS-PAGE and Western blotting. The data were normalized to the levels of β-tubulin, and the expression levels of the different forms of nNOS were confirmed to be similar (WT, 1.52 relative units; 7R, 1.49 relative units; 6R739K, 1.42 relative units; and 6R743K, 1.40 relative units).

**RESULTS**

**Ubiquitination of nNOS by a Purified System Containing CHIP**—As shown in Fig. 1A, incubation of Hsp70-treated nNOS with a ubiquitination system containing purified E1, E2, and CHIP gives a time-dependent formation of higher molecular mass nNOS-bands, which we have previously established as nNOS-ubiquitin conjugates (15). Similar bands have been visualized by anti-ubiquitin IgG after immunoprecipitation with anti-nNOS IgG or with the use of 125I-ubiquitin (15). Based on the estimated molecular mass calculated from the migration of the bands against molecular mass standards, we have labeled the first major band (170 ± 2.3 kDa) above the native nNOS band (161 ± 2.3 kDa) as the mono- ubiquitinated form of nNOS (mono-Ub). The higher mass bands, which appear after longer duration of incubation, are poly-ubiquitinated products (poly-Ub). Although in theory these bands may represent multiubiquitinated products (multiple mono-ubiquitinations), as will be shown below, they are predominantly poly-ubiquitin products. As shown in Fig. 1B, the time-dependent decrease in the nNOS band (closed circles) coincides with the time-dependent increase in the mono-Ub (closed squares).
and poly-Ub (closed triangles) products. The sum of the nNOS and nNOS-ubiquitin bands at each time point (Fig. 1B, open squares) remains the same, suggesting that the majority of the nNOS protein is accounted for in each lane. It is important to note that unlike our previous studies where only a few percent of the total nNOS was ubiquitinated (6, 11, 16), the current system gives a much higher yield (∼50%) of ubiquitinated nNOS. As shown in Fig. 1C, the omission of ubiquitin (lane 2) or CHIP (lane 3) completely blocks the ubiquitination reaction. The use of a mutant ubiquitin (K0R), where the lysines needed to form poly-ubiquitin chains have been mutated to arginine residues, was found to decrease the higher molecular mass nNOS bands, corresponding to both mono- and poly-ubiquitin conjugates. The biologically inactive D-isomer of NNA has no effect (Fig. 2B, lane 4), indicating the stereospecificity of the process. The quantification of the nNOS band clearly shows that L-NNA protects the native enzyme by Hsp70-CHIP (11, 14). A diagnostic test of this mechanism is the decreased nNOS ubiquitination caused by 1-isomer, but not the D-isomer, of \(N^\text{G}-\text{nitro-arginine (NNA)}\) that can be demonstrated in \textit{in vitro} studies with cell extracts, in intact cells, as well as in rats (11, 14, 17). As shown in Fig. 2A, ubiquitination by the purified protein system of nNOS pretreated with 1-NNA (cf. lane 3 with lane 2) decreases the amount of higher molecular mass nNOS bands, corresponding to both poly- and mono-ubiquitin nNOS conjugates. The biologically inactive D-isomer of NNA has no effect (Fig. 2A, lane 4), indicating the stereospecificity of the process. The quantification of the nNOS band clearly shows that 1-NNA protects the native nNOS from ubiquitination (Fig. 2B, nNOS, cf. conditions 2 with 3), whereas the D-NNA has no significant protective effect (Fig. 2B, cf. conditions 2 with 4). Consistent with the effects on the nNOS, there is a significant decrease in the amount of both mono (Fig. 2B, mono-Ub)- and poly (Fig. 2B, poly-Ub)-ubiquiti-
nNOS when l-NNA (condition 3), but not D-NNA (condition 4), is present. Thus, the stereospecific protection by the slowly reversible inhibitor, l-NNA, on nNOS ubiquitination in cells has been faithfully recapitulated by the purified ubiquitination system described here.

Identification of nNOS Ubiquitination Site(s) by LC-MS/MS Analyses—The ubiquitination system described above was scaled up by a factor of 40, and the resulting mixture was filtered through a 100-kDa molecular sieve (Amicon Ultra) to remove unreacted His-ubiquitin and His-CHIP. The His-ubiquitin-nNOS conjugates present in the flow-through fraction were purified by magnetic nickel beads and submitted to SDS-PAGE. The band corresponding to the mono-ubiquitinated nNOS was excised and subjected to in-gel tryptic digestion. The tryptic peptides were analyzed by LC-MS/MS for the diagnostic GG-addition of lysine 739 (nNOS 6R739K) recapitulated the poly-ubiquitination to the level found with the wild type enzyme (Fig. 5B, poly-Ub, cf. solid bars with open bars). We overexpressed and prepared purified 7R nNOS and 6R739K nNOS proteins for in vitro ubiquitination studies with purified proteins. We were unable to obtain purified 6R739K nNOS as overexpression in insect cells produced a clipped form of the protein during expression. Based on the extent of poly-ubiquitination obtained with purified 6R739K nNOS, we did not pursue the preparation of intact 6R743K nNOS further. We did, however, examine the 6R743K nNOS in HEK293T cells as described later. As shown in Fig. 5A, the ubiquitination of the nNOS 7R and 6R739K was compared with the wild type nNOS in the purified ubiquitination system. The polyubiquitination of nNOS 7R mutant is significantly decreased compared with the wild type nNOS (Fig. 5B, poly-Ub, cf. solid bars with open bars), and this could not be explained by an overall decrease in the extent of ubiquitination as evident by the loss of the nNOS to the same levels (Fig. 5B, nNOS, cf. solid bars with open bars). Interestingly, the amount of mono-ubiquitinated nNOS generated from nNOS 7R was unchanged (Fig. 5B, mono-Ub, cf. solid bars with open bars). The reintroduction of lysine 739 (nNOS 6R739K) recapitulated the poly-ubiquitination to the level found with the wild type enzyme (Fig. 5B, poly-Ub, cf. gray bars with open bars).
Role of Calmodulin-binding Region in the Degradation of nNOS—We next examined the proteasomal degradation of the wild type nNOS and nNOS 7R mutant in an \textit{in vitro} system containing fraction II, which is the DE52-retained fraction of reticulocyte lysate that contains ubiquitinating enzymes and the proteasome (Fig. 5, \textit{inset}). We have previously shown that nNOS is proteasomally degraded in an ubiquitin-dependent manner in this system (15). The fraction II-dependent proteasomal degradation is also responsive to the nature of the nNOS heme cleft and dependent on ATP, CHIP, and Hsp70 (11). As shown in Fig. 5C, the nNOS 7R is not degraded after a 3-h incubation (\textit{squares}), whereas /H11011\textit{40\%} of the wild type nNOS (\textit{circles}) is degraded. The nNOS 6R739K (Fig. 5C, \textit{triangles}) was proteasomally degraded by fraction II to a level intermediate between wild type and nNOS 7R.

We also examined the CHIP-dependent proteasomal degradation of nNOS in intact HEK293T cells (Fig. 6A). We have previously established that CHIP overexpression decreases the levels of nNOS in HEK293T cells (6). As shown in Fig. 6B, the quantification of nNOS clearly shows that the decrease in nNOS caused by CHIP (\textit{closed circles}) is greatly attenuated by lactacystin (\textit{closed squares}), indicating that CHIP causes the proteasomal degradation of nNOS. This is consistent with that found previously (6). As shown in Fig. 6C, we have examined the 7R, 6R739K, and 6R743K nNOS mutants under the same
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FIGURE 5. Effect of mutations in the CaM-binding region on in vitro ubiquitination and degradation of nNOS. A, ubiquitination of nNOS, nNOS 7R, and nNOS 6R739K by the purified ubiquitination system. Samples were incubated for 1 h and analyzed by Western blotting as described under “Experimental Procedures.” nNOS starting material (nNOS), mono-ubiquitinated nNOS (mono-Ub), and poly-ubiquitinated nNOS (poly-Ub) are indicated. B, amount of mono-ubiquitinated (mono-Ub) and poly-ubiquitinated (poly-Ub) nNOS from A was quantified. The residual amount of nNOS was also measured (nNOS). nNOS, closed bars; nNOS 7R, open bars; nNOS6R739K, gray bars. The values are the mean ± S.E. (n = 6). Statistical probability is expressed as follows: *, p < 0.05; **, p < 0.01. C, degradation of WT nNOS, nNOS 7R, and nNOS 6R739K by fraction II. The nNOS was treated with fraction II, and the proteasomal degradation of nNOS was assessed by Western blotting as described under “Experimental Procedures.” Samples were collected at the indicated time points and analyzed by Western blot quantification of the bands shown. nNOS, circles; nNOS 7R, squares; nNOS 6R739K, triangles. The values are the mean ± S.E. (n = 3). Statistical probability is expressed as follows: *, p < 0.05; **, p < 0.01. The inset shows quantification of nNOS bands under the following conditions: complete system (Complete), complete system plus 10 μM MG132 (+MG132), and complete system without ubiquitin (−Ub).

Discussion

We show here that the CHIP-mediated ubiquitination of nNOS under in vitro conditions occurs primarily on the oxygenase domain of the enzyme. Of 12 identified ubiquitination sites, nine are located in the oxygenase domain, two are located in the calmodulin-binding region, and one is located in the reductase domain. Unfortunately, there is no known crystal structure of the full-length nNOS, but we have adapted a diagram of nNOS by Garcin et al. (28) to depict the location of the ubiquitination sites. As shown in Fig. 7, one monomer of nNOS is depicted in blue and the other in gray. In Fig. 7, the oxygenase domain (Oxy) is shown with an N terminus (N) placed near the dimer interface based on the location of the most N-terminal residue that has been defined in the oxygenase crystal structure (29). The C terminus of the oxygenase domain is connected to the calmodulin-binding domain (Fig. 7, CaM) that links the oxygenase domain to the reductase domain (Red). The ubiquitination sites appear to be primarily on the oxygenase and calmodulin domains. Based on the primary sequence, the ubiquitination sites in the calmodulin domain border the reductase domain. It is noteworthy that the Hsp70-CHIP complex has been shown to recognize primarily the oxygenase domain and that this binding is responsive to the conformation of the heme-binding cleft (18). The sites of ubiquitination are consistent with the notion that Hsp70 recognizes and binds the heme-binding cleft and directs the CHIP-dependent ubiquitination at nearby sites on nNOS (17).

The biological role of the multiple ubiquitination sites found from the in vitro studies is unclear. However, previous studies have demonstrated the ability of calmodulin, in the presence of Hsp90, to block nNOS ubiquitination (16). Moreover, Hsp90 is known to enhance the binding of calmodulin (30), and Hsp90 is known to protect nNOS from ubiquitination (9). Thus, our initial attempts focused on determining the functional significance of the ubiquitination sites within the calmodulin-binding domain. We employed a common strategy to account for the known promiscuity of ubiquitination by use of a mutant (nNOS 7R) where all seven lysines in the calmodulin-binding domain of nNOS were replaced with arginines, which are not able to conjugate to ubiquitin. The nNOS 7R was not poly-ubiquitinated nor degraded in vitro and was not degraded after overexpression of CHIP in intact cells. In contrast, knocking back in the lysine at residue 739 of the nNOS 7R recapitulated the polyubiquitination and degradation of the nNOS. Thus, we conclude that lysine residue 739 of nNOS is a major site of polyubiquitination and signals for proteasomal degradation of the enzyme. Interestingly, the in vitro mono-ubiquitination of the nNOS 7R mutant was similar to the wild type enzyme and indicates that sites apart from those in the calmodulin-binding site account for the mono-ubiquitination of nNOS. The mono-ubiquitination is a known signal for receptor internalization and endocytic degradation (27). The exact role of mono-ubiquitination of nNOS is not known, although this form is found in relatively high abundance in cells and in cytosolic preparations of rat brain (15).

Although many protein substrates for CHIP-dependent ubiquitination have been identified, there are only a few studies where the actual sites of ubiquitination have been characterized either by mutational analysis (31) or by LC-MS/MS methods (32–34), and to our knowledge there are none where both approaches were used, as in this study. Correia and co-workers (33, 34) have shown that CHIP ubiquititates CYP2E1 and CYP3A4 in vitro at multiple sites as determined by LC-MS/MS analyses, but no site was examined by mutational methods. Interestingly, the ubiquitination sites appeared to co-cluster with phosphorylation sites, and the notion of a phosphodegron for P450 degradation has been raised (33, 34). We do not know if phosphorylation triggers the ubiquitination of nNOS. However, it is noteworthy that an affinity-purified antibody directed conditions. Unlike in insect cells, the 6R743K nNOS was not appreciably clipped, and the intact form was blotted and quantified. As shown in Fig. 6B, the 7R nNOS (open circles) were not significantly changed by CHIP overexpression. However, the 6R739K nNOS mutant (Fig. 6D, open squares) was significantly decreased by CHIP overexpression to a level that was similar to the wild type nNOS. The amount of 6R739K and wild type nNOS were not significantly different (p > 0.05) at any level of CHIP.

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Although many protein substrates for CHIP-dependent ubiquitination have been identified, there are only a few studies where the actual sites of ubiquitination have been characterized either by mutational analysis (31) or by LC-MS/MS methods (32–34), and to our knowledge there are none where both approaches were used, as in this study. Correia and co-workers (33, 34) have shown that CHIP ubiquititates CYP2E1 and CYP3A4 in vitro at multiple sites as determined by LC-MS/MS analyses, but no site was examined by mutational methods. Interestingly, the ubiquitination sites appeared to co-cluster with phosphorylation sites, and the notion of a phosphodegron for P450 degradation has been raised (33, 34). We do not know if phosphorylation triggers the ubiquitination of nNOS. However, it is noteworthy that an affinity-purified antibody directed
against a highly conserved domain comprising the lysine residue 317 to alanine residue 340 of CYP2E1 inhibited both catalytic activity and ubiquitination of the P450 (35). Thus, once again there is a link between the heme-binding cleft and ubiquitination. The authors speculated that this domain likely explains the substrate stabilization of CYP2E1 (35). The importance of the cleft is further supported by the discovery that CHIP mediates ubiquitination of CYP2E1 and that ubiquitination is enhanced after suicide inactivation of the P450 (36, 37). Thus, it is highly likely that the heme cleft signals for ubiquitination similar to that found for nNOS. We do not rule out the possibility that cleft opening signals for phosphorylation of P450 enzymes, including nNOS.

In this study, we have characterized the sites of CHIP-dependent ubiquitination on nNOS and identified lysine 739 as the major site for poly-ubiquitination and signal for degradation in cells. The site of poly-ubiquitination is important in understanding how Hsp70 CHIP regulates nNOS degradation. Drugs and other xenobiotics greatly impact the degradation of nNOS either by enhancing turnover after suicide inactivation or by decreasing turnover when the natural substrate L-arginine or the slowly reversible inhibitor NG-nitro-L-arginine is present (14). D-Arginine or NG-nitro-D-arginine do not decrease turnover of nNOS indicating stereoselectivity of the process. The exact mechanism for these effects is not known, although they clearly involve interactions at the heme active site and Hsp70 CHIP (16). Chaperones regulate client proteins through interactions with ligand-binding clefts, and we have proposed that the conformation about the heme-binding cleft of nNOS is sensed by chaperones to selectively cull altered forms of nNOS for degradation (17, 38). Consistent with this notion, NG-nitro-L-arginine decreases the amount of Hsp70 and CHIP bound to nNOS, whereas the NG-nitro-D-arginine has no effect (18). Accordingly, NG-nitro-L-arginine decreases ubiquitination of nNOS in a variety of in vitro and cell systems, decreases the

**FIGURE 6.** CHIP-dependent proteasomal degradation of nNOS in HEK293T cells. HEK293T cells were co-transfected with various forms of nNOS cDNA (0.5 µg) and various amounts of CHIP cDNA (0, 0.1, 0.5, and 1 µg) for 48 h and treated with lactacystin (10 µM) for the last 20 h as described under “Experimental Procedures.” A, CHIP-dependent proteasomal degradation of nNOS. nNOS was co-transfected with increasing amounts of CHIP as indicated. The cells were treated in the absence (lanes 1–4) or presence (lanes 5–8) of lactacystin (10 µM) as indicated. B, quantification of nNOS amounts from data depicted in A. WT nNOS (circles) and WT nNOS treated with lactacystin (squares) are shown. The values are the mean ± S.E. (n = 3). Statistical probability is expressed as follows: **, p < 0.01; ***, p < 0.001. C, effect of CHIP on nNOS 7R (lanes 1–4), 6R739K (lanes 5–8), and 6R743K (lanes 9–12) levels. nNOS 7R, nNOS 6R739K, and nNOS 6R743K were co-transfected with increasing amounts of CHIP and blotted as in A. D, quantification of nNOS amounts from data depicted in C. nNOS 7R (triangles), nNOS 6R739K (open squares), and nNOS 6R743K (open circles) are shown. The values are the mean ± S.E. (n = 3). Statistical probability is expressed as follows: *, p < 0.05; ***, p < 0.001.

**FIGURE 7.** Schematic of the ubiquitination sites on the structure of nNOS. Schematic drawing of full-length nNOS, adapted from Garcin et al. (28). The full-length structure is not known, but based on known biochemical data as well as studies of the flow of electrons through the enzyme, Garcin et al. (28) depicted the oxygenase domain above the reductase domain, with the calmodulin-binding domain in the middle. One monomer is shown in blue and one in gray. The oxygenase (Oxy) and reductase (Red) domains are indicated. The calmodulin-binding domain (CaM) is indicated and shown connected to the nNOS oxygenase and reductase domains by a dashed line. All 12 ubiquitination sites are indicated. The N (N) and C termini (C) of the oxygenase domains are indicated.
turnover of the protein in cells, and increases the amount of nNOS protein in vivo (11, 14, 16, 18, 39). Defining the sites of ubiquitination should ultimately provide a framework for understanding how the chaperones and CHIP recognize and bind to nNOS to ubiquitinate lysine residue 739 as a signal for degradation of nNOS to maintain protein quality control. It is intriguing to think that ligands that alter the conformation of the active site of nNOS could be developed to alter the amount of nNOS through alteration of chaperone-mediated degradation. The sites of mono-ubiquitination are also regulated by the active site conformation, but the functional role for these modifications is currently not known. Future studies will be directed at understanding the role of mono-ubiquitination in the regulation of nNOS.

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