Chaperone-dependent Ubiquitination of Neuronal Nitric Oxide Synthase

by

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ABSTRACT

Nitric oxide synthase (NOS), a cytochrome P450-like hemoprotein enzyme, catalyzes the synthesis of nitric oxide, a critical signaling molecule in a variety of physiological processes. Our lab has found that certain drugs, such as guanabenz, inactivate neuronal NOS (nNOS) and lead to the ubiquitination of the nNOS. To better understand the molecular trigger for nNOS ubiquitination, we characterized the proteins that are involved in nNOS ubiquitination, examined a mutant nNOS that can serve as a model for inactivated nNOS, and identified the sites of ubiquitin attachment to nNOS. Using an *in vitro* model for ubiquitination containing Fraction II, the DE52-retained fraction of reticulocyte lysates that contains all ubiquitinating enzymes and the proteasome, we found that CHIP (C-terminus of Hsp70-interacting protein) and Hsp70 play a major role in promoting the ubiquitination of nNOS, whereas Hsp90, in concert with calmodulin, protects nNOS from ubiquitination. We found a C331A nNOS mutant that is highly susceptible to CHIP-dependent ubiquitination in cells and *in vitro* systems. Substrates and other ligands stabilize the C331A nNOS against ubiquitination, suggesting that subtle alterations to the active site cleft are sufficient for triggering the ubiquitination. The C331A nNOS is ubiquitinated in an Hsp70/CHIP-dependent manner, similar to the wildtype enzyme. With the use of an *in vitro* system containing purified proteins, including E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase CHIP, we recapitulated the ubiquitination of nNOS seen in cells. We identified the sites of ubiquitination on nNOS through LC-MS/MS analysis of the ubiquitinated nNOS obtained from the *in vitro* purified system. Of the twelve sites that

were identified, nine are located in the oxygenase domain, two in the calmodulin-binding region, and one in the reductase domain of the enzyme. These data are consistent with studies showing that the oxygenase domain and the calmodulin-binding domain play a major role in regulating the ubiquitination of nNOS. Thus, alterations to the heme active site structure, whether by drug-mediated inactivation or the destabilizing C331A mutation, leads to ubiquitination by Hsp70 and CHIP in the calmodulin-binding region and/or the oxygenase domain of nNOS.

CHAPTER 1

INTRODUCTION

Nitric Oxide

Nitric oxide (NO) is a small radical that readily diffuses along concentration gradients across cell membranes and between cells. As a result, NO is both an intracellular and an intercellular signaling molecule. The biological effects of NO were first described in 1980 by Furchgott and Zawadzki (1), who demonstrated its role in endothelial relaxation. Since then, a role for NO in a wide variety of physiological effects has been described, including regulation of blood pressure, blood clotting, immune response, and neurotransmission. Pure NO is a gas under standard temperature and pressure, but it exists as a dissolved nonelectrolyte under most biological conditions. Due to its radical nature, NO is highly reactive, and thus has a short half-life of 5-10 seconds *in vitro* (2).

As NO contains one unpaired electron, its most common chemical interactions include stabilization of the unpaired electron (2). This can occur through reactions between NO and other paramagnetic species such as oxygen, superoxide, and peroxy radicals, or by forming an NO-metal complex. Two molecules of NO can react with oxygen to form two molecules of the paramagnetic radical nitrogen dioxide. Nitrogen

dioxide can then react further to produce dinitrogen tetroxide or dinitrogen trioxide, both of which are highly reactive. One molecule of NO will react extremely rapidly with superoxide to produce the reactive species peroxynitrite. Peroxynitrite is a potent oxidant, capable of oxidizing thiols (3) and DNA bases (4), and it can initiate metal-independent lipid peroxidation (5). Additionally, NO is structurally similar to dioxygen, so proteins that bind dioxygen as part of their normal function, such as hemoglobin, myoglobin, nitric oxide synthase (NOS), and the cytochrome P450s, are susceptible to inhibition by NO (2). Conversely, NO binds the heme iron in soluble guanylate cyclase, thus activating the enzyme by causing a conformational change in the protein that increases the rate of cyclic GMP formation (6).

Nitric Oxide Formation

Nitric oxide is synthesized from the amino acid L-arginine by the enzyme nitric oxide synthase. This enzyme catalyzes the five-electron oxidation of the guanidine nitrogen of L-arginine in the presence of molecular oxygen to form NO and L-citrulline (Figure 1.1). The reaction also depends on the availability of several required cofactors, including (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin (CaM), and iron protoporphorin IX (heme), as well as nicotinamide adenine dinucleotide phosphate (NADPH) as an electron source (7). The electrons from NADPH are transferred through the flavins, FAD and FMN, to the heme active site, where O_2 is activated and L-arginine is oxidized. Bound calmodulin facilitates the flow of electrons from the flavins to the heme (8). N-hydroxy-arginine is an intermediate in the formation of NO (9).

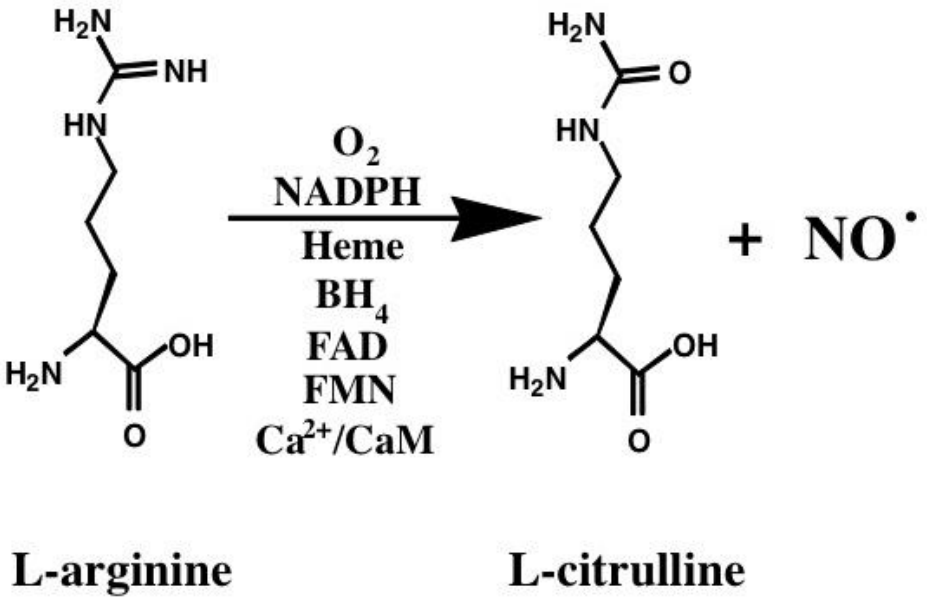

Figure 1.1 Reaction catalyzed by NOS

Structure of Nitric Oxide Synthase

NOS is a multi-domain enzyme that is catalytically active only as a homodimer. Each monomer consists of two domains, an N-terminal oxygenase domain, and a C-terminal reductase domain (Figure 1.2). The domains are linked by a calmodulin binding region consisting of 30 amino acids (7, 8). The reductase domain contains binding sites for NADPH and the flavins, FAD and FMN, and it is able to function as a monomer (10). Additionally, the reductase domain shares strong sequence similarity with cytochrome P450 reductase (10). The oxygenase domain contains binding sites for the heme, BH_4, and the substrate, L-arginine, all of which are required for activity of the enzyme (11). The heme group is coordinated to the oxygenase domain through a cysteine thiolate, as in cytochrome P450 enzymes, and it has a reduced CO difference spectrum with a Soret band at approximately 450 nm (7, 12). NOS produces NO only as a dimer. Dimerization

can occur with the oxygenase domains alone, as the reductase domain does not contain any of the essential determinants for dimerization. The dimerization of iNOS is promoted by heme incorporation, BH_4, and L-arginine (7), whereas eNOS and nNOS dimer assembly requires heme incorporation (13), but may not require BH_4, although BH_4 is able to stabilize the nNOS dimer once it is formed (14). Crystal structures of iNOS and eNOS isoforms have revealed that the monomers align in a head-to-head manner (15-17) and a 55 amino acid sequence in the oxygenase domain has been identified in iNOS as critical for dimerization (18). The flow of electrons during the catalytic cycle of NOS occurs from the reductase of one monomer subunit to the oxygenase domain of the other monomer (19), which highlights the importance of dimerization in NOS function.

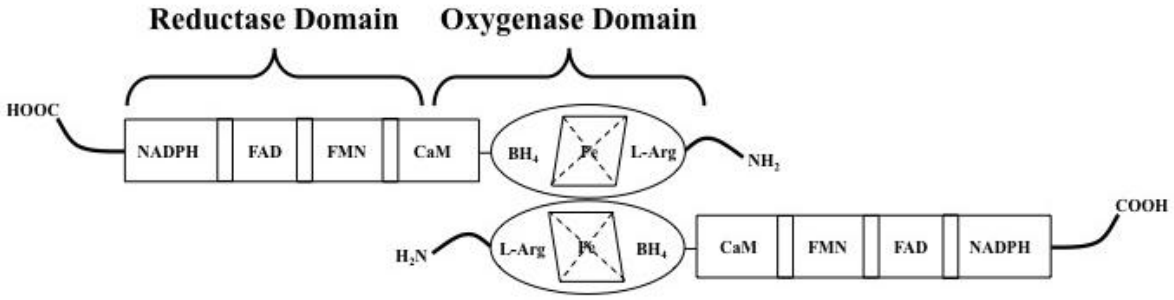

Figure 1.2 NOS domains and structure

NOS Isoforms

Three main isoforms of NOS have been identified: isoform I (neuronal NOS, nNOS), which is constitutively expressed in a variety of neuronal cells; isoform II (inducible NOS, iNOS), which is usually not constitutively expressed but can be induced

in macrophages and other cells; and isoform III (endothelials NOS, eNOS), which is expressed in endothelial cells (20). The isoforms share approximately 60% amino acid identity and have highly similar structures. The three isoforms have regions of high homology, however there are some significant differences between them. For example, iNOS expression can be induced by various inflammatory stimuli such as cytokines or bacterial lipopolysaccharides (21, 22). In contrast, eNOS and nNOS are not primarily regulated at the transcriptional level. Rather, they are regulated by changes in the level of intracellular calcium, with both isoforms requiring a bound Ca^{+2}/CaM complex for activation. Both isoforms are inactive at 100 nmol/L Ca^{+2} and fully active at 500 nmol/L, which are concentrations of Ca^{+2} typically seen before and after receptor stimulation of excitatory cells (23, 24). In contrast, iNOS also requires bound Ca^{+2}/CaM, but its affinity for CaM is so great that it is always bound and is therefore not influenced by levels of intracellular Ca^{+2} (25). There is also a size variation between the isoforms, with iNOS and eNOS having masses of 130 kDa and 134 kDa, respectively, while nNOS is somewhat larger at 160 kDa, due to an extra 250 amino acid sequence containing a PDZ domain at its amino terminus, which is involved in the cellular targeting of this isoform (26, 27). eNOS contains N-terminal myristoylation and palmitoylation sites that regulate its localization to plasmalemmal caveolae (28).

Physiological Roles of NO

NO is involved in many important physiological functions. NO formed by nNOS in neurons acts as a neurotransmitter in the central and peripheral nervous systems. In the central nervous system, NO can be involved in processes such as pain perception,

neuronal plasticity, memory formation, and weight and appetite control (26, 29). In the peripheral nervous system, NO acts as an inhibitor of non-adrenergic, non-cholinergic nerves that relax smooth muscles in the gastrointestinal, respiratory, vascular, and urogenital systems (29). Alternate splice variants of nNOS are also found in penile tissue and skeletal muscle (30). NO released by endothelial cells acts as a regulator for the vascular system, causing vascular smooth muscle relaxation through its interaction with soluble guanylate cyclase (2). Endothelium-derived NO can also regulate platelet aggregation and cardiac load (31). iNOS is expressed primarily in cells of the immune system, such as macrophages, but can also be found in astrocytes, chondrocytes, hepatocytes, and myocytes (32). NO produced by iNOS is involved in host-defense mechanisms to eliminate invading parasites and microbes, as well as playing a role in wound healing by promoting collagen synthesis and angiogenesis (33, 34).

Pathophysiology of NO

Although NO plays a role in many important physiological processes, changes in the amount of NO produced can result in pathology. Overproduction of NO or the superoxide ion as a result of nNOS dysfunction is associated with several neurodegenerative disorders, including Huntington's disease, ischemia, stroke, Alzheimer's disease, migraines, schizophrenia, Parkinsons's disease, and multiple sclerosis (26, 35-37). Excessive production of NO by iNOS is involved in cerebral ischemia and stroke, as well as septic shock (38, 39). Chronic overexpression of iNOS may be involved in autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease, diabetes, psoriasis, and myocardial dysfunction (34, 40, 41).

Underproduction of NO has been implicated in the etiology of several diseases as well. Deficiency of NO plays an important role in models for hypertension and atherosclerosis (33, 42). Patients with diabetes and the elderly are known to have an increased incidence of impotence that is paralleled with a loss of penile NOS activity (43, 44). Impotence is a common side effect in patients taking guanabenz. When administered to rats, guanabenz was shown to inactivate nNOS and cause the loss of nNOS protein in penile tissue (45-47). Cigarette smoke has been shown to be an inhibitor of nNOS, which accounts for the high prevalence of impotence, hypertension and cardiovascular disease seen in smokers (48, 49).

NOS as a Therapeutic Target

Since disruptions in the normal production of NO have been implicated in many disease states, NOS is a potential therapeutic target for controlling NO production. Impaired NOS function seen in smokers and patients with Type II diabetes has been ameliorated by treatment with the NOS cofactor BH_4 (50, 51). Similarly, administration of the NOS substrate, L-arginine, has been used to treat decreased NO production seen in conditions such as pulmonary hypertension and ischemia-reperfusion injury (52, 53).

NOS inhibitors may be useful for conditions where excessive NO production is causing a specific pathology. Most inhibitors are analogues of the substrate L-arginine, and are able to bind to the catalytic site of NOS. Of these analogues, there are two types of inhibitors: reversible inhibitors and irreversible inactivators. Reversible inhibitors simply compete with the substrate for binding in the active site of NOS to cause loss of activity. Examples of this type of inhibitor include N^G-methyl-L-arginine and the slowly

reversible inhibitors N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester (2). Irreversible (metabolism-based) inactivators are for the most part similar in structure to the substrate, and they are metabolized by the enzyme to a highly reactive intermediate that can then covalently modify and ultimately inactivate the enzyme (54). The covalent modification seen with these compounds is irreversible, giving this class of compounds the nickname of suicide inactivators, since the enzyme catalyzes its own demise. This type of compound has also been studied extensively with the cytochrome P450 enzymes (55). Compounds such as aminoguanidine, N^G-methyl-L-arginine, and guanabenz are examples of suicide inactivators of NOS (56). Another group of NOS inhibitors are those that compete for binding at both the substrate and BH_4 binding site (57). These compounds are often similar in structure to BH_4, and include compounds such as 7-nitroindazole (57).

Post-Translational Regulation of NOS

NO cannot be stored, released, or inactivated like classical neurotransmitters (58), and because of its potent chemical reactivity and high diffusibility, the regulation of NOS is extremely important for regulating levels of NO. There are many post-translational mechanisms for regulating NOS, including lipid modifications, phosphorylation, and interactions with various proteins (59). eNOS cellular localization can be regulated by myristoylation, palmitoylation, farnesylation, and acetylation within the oxygenase domain (60). eNOS and nNOS are both phosphorylated in their oxygenase domains (61, 62). A variety of proteins have been shown to interact with NOS enzymes, such as PIN (protein inhibitor of nNOS) which interacts with the PIN binding domain of nNOS and is

believed to be involved in destabilizing the dimer (63), CAPON (carboxy-terminal PDZ ligand of nNOS), which interacts with the PDZ domain of nNOS and can restrict NO generation (64), calmodulin (65), caveolin-1 and caveolin-3, which affects localization of eNOS within caveolae (66).

The NOS enzymes are also regulated by protein chaperones. Hsp90 (heat-shock protein 90) enhances the activity of all three NOS isoforms, as was shown in intact cells (67-70) and by activation assays with purified proteins (71-73). NOS activity is Ca^{2+}/CaM-dependent, and several signaling pathways initiate nNOS and eNOS activity by raising intracellular Ca^{2+} concentrations. Studies with purified proteins show that CaM and Hsp90 increase the binding of each other to both eNOS and nNOS (71-74). Hsp90 also protects nNOS from proteasomal degradation. In the presence of the Hsp90 inhibitor geldanamycin, which blocks hsp90 function, nNOS protein levels decreased by 50% in HEK293 cells, due to enhanced turnover of NOS (68). Additionally, the NOS activity in these cells was reduced by 50% (68), implying that hsp90 is important for both nNOS stability and function in the intact cell.

In contrast to the stabilizing action of Hsp90, Hsp70 (heat-shock protein 70), and its cochaperone Hsp40 (heat-shock protein 40) are involved in the ubiquitination of many proteins, including nNOS (75-77). There is considerable evidence that Hsp70 promotes degradation of proteins, including nNOS, by promoting ubiquitination mediated by chaperone-dependent E3 ubiquitin ligases (78).

Ubiqutination and Degradation of NOS

The ubiquitin-proteasome system is the major degradation pathway for short-lived, regulatory proteins such as NOS. In this pathway, a dysfunctional or altered protein is selectively recognized for ubiquitination, a process whereby an ubiquitin molecule is conjugated to a lysine residue by three ubiquitinating enzymes — E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzyme, and E3 ubiquitin ligase (Figure 1.3) (79). This process repeats, leading to the formation of a polyubiquitin chain, which then targets the protein for proteolysis by the 26S proteasome, where it is hydrolyzed to small peptides and the ubiquitin is recycled (79, 80). There is one known E1 enzyme, whereas there are several E2 enzymes and a large number of E3 enzymes (81). The E3 ligases can be separated into three main groups based on their mechanisms of action: a covalent mechanism (Homologous to E5AP C-Terminus (HECT)-domain E3s), a non-covalent mechanism (Really Interesting New Gene (RING) finger E3s), and a chaperone dependent mechanism (U-box E3s) (81). An example of the latter is CHIP (C-terminus of hsp70-interacting protein), which is an E3 ligase for nNOS that interacts through a tetratricopeptide repeat (TPR) domain with both hsp70 and hsp90 (82, 83). Although I will show in Chapter II that CHIP is the major E3 ligase for nNOS in a physiological system, Parkin is another Hsp70-dependent E3 ligase that has been shown to ubiquitinate nNOS in CHIP knockout cells (78).

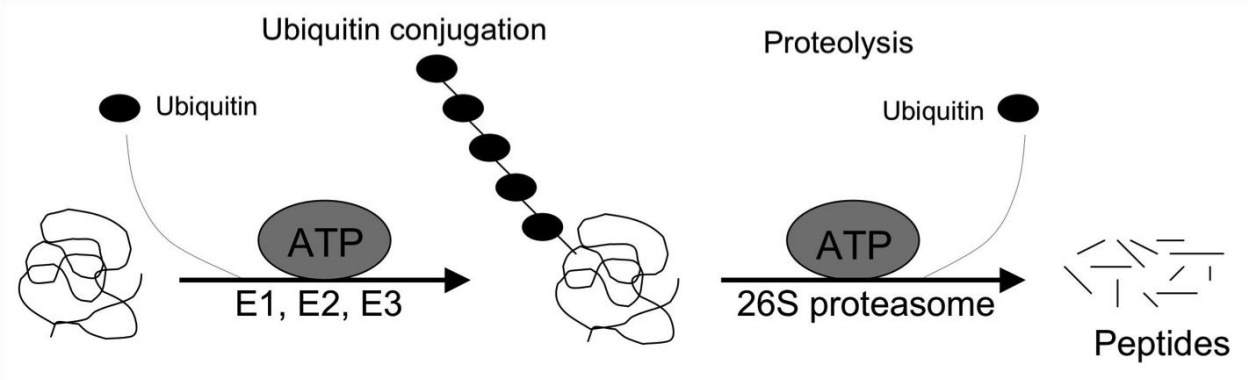

Figure 1.3 Ubiquitin-proteasome system

Recognition of substrates for ubiquitination is a highly selective process that depends on the availability of an ubiquitinatable lysine residue as well as the appropriate E3 ligase (84). Specific protein motifs that are known to trigger ubiquitination include the N-end rule, in which specific N-terminal residues stimulate rapid polyubiquitination and degradation *in vivo* (85); a conserved carboxy-terminal domain in the yeast protein Rpb1 (RNA polymerase II) which contains a heptapeptide repeat sequence SPTSPSY that is recognized by the E3 ligase Rsp5 (86); and a proline-rich motif in the human epithelial sodium channel that, when mutated, impairs its degradation in the heritable disorder Liddle's syndrome (87). Other examples of triggers for ubiquitination include phosphorylation of the N-terminal regulatory domain of NF-kB (84) and exposure of hydrophobic protein surfaces seen with the yeast transcription factors MATα1 and MATα2 (84).

In the case of nNOS, exposure of hydrophobic residues in the heme active site cleft is proposed as a possible trigger for the ubiquitination and degradation of the enzyme (75). The recognition of hydrophobic regions by molecular chaperones may be used for protein quality control of misfolded or abnormal proteins. It has been shown, for

example, that suicide inactivation of nNOS enhances its degradation (47), and that overexpression of CHIP enhances ubiquitination of nNOS in HEK293 cells (75). CHIP has also been shown to ubiquitinate iNOS (88). As an alternative role in protein quality control, CHIP has been shown to play a role in the cellular localization of eNOS, by redistributing eNOS to inactive detergent-insoluble pools, when Hsp90 is inhibited (89).

Another known trigger for nNOS ubiquitination is destabilization of the dimer. The inactive, monomeric form of nNOS is preferentially ubiquitinated and degraded over the enzymatically active homodimer (90, 91). Moreover, the slowly reversible inhbitor N^G-nitro-L-arginine can stabilize and protect nNOS from degradation (91). Tetrahydrobiopterin depletion is another method by which nNOS ubiquitination and degradation is enhanced. An inhibitor of the *de novo* synthesis of BH_4, 2,4-diamino-6-hydroxypyrimidine, decreased the total amount of nNOS dimer and enhanced ubiquitination (92). Additionally, supplementing *in vitro* ubiquitination and degradation of nNOS with BH_4 protects it from both ubiquitination and degradation (92). Moreover, guanabenz-induced depletion of BH_4 and enhanced degradation can be prevented with supplementation of BH_4 (93).

Thesis Rationale

In addition to enhancing ubiquitination of nNOS in HEK293 cells, CHIP has been found in heterocomplexes with Hsp70 coimmunoabsorbed with nNOS from HEK cytosol (75). Also, in a purified system containing nNOS, E1, E2, and CHIP, directed ubiquitination of nNOS occurred in a manner that was enhanced by Hsp70 (75). However, it is unknown what role Hsp70 and CHIP play in the ubiquitination of nNOS in

more physiologically relevant models of ubiquitination. Because CHIP also interacts with Hsp90 by binding to the TPR acceptor site with the TPR domain of CHIP (82, 83), it is possible Hsp90 may also play a role in the ubiquitination of nNOS. Thus, Chapter II focuses on determining the effects of Hsp90 and Hsp70 on nNOS ubiquitination by a physiological ubiquitination system.

A possible trigger for ubiquitination after suicide inactivation of nNOS was proposed to be the exposure of hydrophobic surfaces in the active site cleft (75). It is not known whether the covalent modifications within the cleft lead to a more global unfolding of the enzyme, or if subtle alterations to the active site cleft are sufficient for triggering the ubiquitination of nNOS. In Chapter III, I studied the ubiquitination of a mutant form of the enzyme that serves as a tool for understanding the labilization of nNOS. This mutant, the C331A nNOS mutant, has decreased substrate binding, but is otherwise intact and is enzymatically active (94). However, as I show in this chapter, it undergoes more rapid proteasomal degradation than wild-type nNOS.

Identification of the site(s) of ubiquitination is an important step in determining the process by which a protein becomes recognized by chaperones and then targeted to the ubiquitin-proteasome system for degradation. Studies have indicated the ability of Hsp90 and calmodulin to block the ubiquitination of nNOS *in vitro* (95), however it is unknown where the exact site(s) of ubiquitination of nNOS occurs. Chapter III investigates the site(s) of ubiquitination of nNOS. As I show in this chapter, the oxygenase and calmodulin-binding domains play important roles in regulating nNOS turnover.

The Specific Aims of my thesis are:

I. **To determine the role of Hsp90 and Hsp70 on nNOS ubiquitination by a physiological ubiquitination system.**

II. **To examine the ubiquitination of a C331A mutant of nNOS as a model for suicide-inactivated nNOS.**

III. **To determine the site(s) of ubiquitination of nNOS.**

These studies will address how nNOS becomes a substrate for ubiquitination and proteasomal degradation, and investigate the proteins that are involved in the targeting of nNOS for this process. Understanding the process by which dysfunctional proteins are selectively culled for ubiquitination is important for determining the ultimate physiological effects of drugs and other xenobiotics that can produce dysfunctional proteins and help predict potential drug toxicities.

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CHAPTER 2

OPPOSING ACTIONS OF HSP90 AND HSP70 ON THE UBIQUITINATION OF NEURONAL NITRIC OXIDE SYNTHASE

Summary

NO production by neuronal nitric oxide synthase (nNOS) requires calmodulin and is enhanced by the chaperone Hsp90, which cycles dynamically with the enzyme. The proteasomal degradation of nNOS is enhanced by suicide inactivation and by treatment with Hsp90 inhibitors, the latter suggesting that dynamic cycling with Hsp90 stabilizes nNOS. Here, I use the classic ubiquitinating system, prepared by DE52 chromatography of reticulocyte lysate, to show that Hsp90 inhibits nNOS ubiquitination. Like the established Hsp90 enhancement of NO synthesis, Hsp90 inhibition of nNOS ubiquitination is Ca^{2+}/calmodulin-dependent, suggesting that the same interaction of Hsp90 with the enzyme is responsible for both enhancement of nNOS activity and inhibition of ubiquitination. I also show that methylene blue, an inhibitor of Hsp70 ATPase activity, inhibits nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate, and the block in ubiquitination is overcome by addition of purified Hsp70. Additionally, nNOS ubiquitination is inhibited by anti-CHIP serum. This suggests that Hsp70-directed CHIP E3 ligase activity is responsible for nNOS ubiquitination in this system. Thus, the two chaperones in the Hsp90/Hsp70-based

chaperone machinery, have opposing actions on nNOS ubiquitination, with Hsp70 stimulating and Hsp90 inhibiting. We envision that as nNOS undergoes toxic damage, the heme/substrate binding cleft opens, exposing hydrophobic residues as the initial step in unfolding. As long as Hsp90 can form even transient complexes with the opening cleft, ubiquitination by Hsp70-dependent E3 ligases, like CHIP, is inhibited. When unfolding of the cleft progresses to a state that cannot cycle with Hsp90, Hsp70-dependent ubiquitination is unopposed. In this way, the Hsp70/Hsp90 machinery makes the quality control decision for stabilization versus degradation of nNOS.

Introduction

Both the function and turnover of a wide variety of signaling proteins, such as steroid receptors and protein kinases, are regulated by Hsp90 (reviewed in Ref. 1). These Hsp90 'client' proteins are assembled into complexes with the chaperone by a multichaperone machinery in which Hsp90 and Hsp70 function together as essential components (1). Formation of heterocomplexes with Hsp90 stabilizes client proteins, and treatment with an Hsp90 inhibitor such as geldanamycin uniformly triggers their degradation (2). Degradation of the Hsp90-regulated signaling proteins occurs via the ubiquitin-proteasome pathway, which in this case is initiated by Hsp70-dependent E3 ubiquitin ligases, such as CHIP (3) and parkin (4).

The interaction with Hsp90 modulates the ligand binding clefts in client signaling proteins to increase the efficiency of binding of ligands, such as steroids or ATP (reviewed in Refs. 5 and 6), and the proteins constantly undergo cycles of Hsp90 heterocomplex assembly and disassembly in the cytoplasm and nucleoplasm (1). Two

types of cycling with Hsp90 have been recognized. The classical client signaling proteins form Hsp90 heterocomplexes that are stable enough to be isolated and analyzed biochemically. For lack of a better term, we call this 'stable cycling' with Hsp90, and these proteins are stringently regulated by the chaperone (6). In contrast, other signaling proteins form Hsp90 heterocomplexes that rapidly disassemble such that no (or only trace amounts of) Hsp90 heterocomplexes are recovered from cell lysates. We call this 'dynamic cycling', and the activity and turnover of these proteins are not as affected by Hsp90 inhibitors as the classical client proteins (5,6).

The nitric oxide synthases (NOSs), including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), are signaling proteins whose activity is enhanced by Hsp90, as shown both by studies in intact cells (7–10) and by direct activation assays with purified proteins (7,10–14). NOS activity is Ca^{2+}/calmodulin (CaM)-dependent, and several signaling pathways initiate nNOS and eNOS activity by raising intracellular Ca^{2+} concentration. Studies with purified proteins show that CaM and Hsp90 increase the binding of each other to both eNOS and nNOS (11,12,14,15). Both direct binding of purified Hsp90 to purified eNOS and nNOS and activation of their activities have been demonstrated in the absence of ATP and Hsp70 (7,14,15). This stands in contrast to the assembly of stable Hsp90 complexes with steroid receptors, for example, where both receptor-bound Hsp70 and Hsp90 must pass through at least one complete ATPase cycle (1). Thus, the observations made with Hsp90 regulation of eNOS and nNOS differ from the classic client signaling proteins, yet, there are similarities between them.

For example, formation of Hsp90 heterocomplexes with the glucocorticoid receptor (GR), the most studied client protein, promotes high affinity ligand binding, thus facilitating response at low concentrations of steroid (1). Similarly, in Sf9 insect cells, which have a low level of endogenous heme, Hsp90 promotes heme binding by apo-nNOS, with concomitant conversion to the enzymatically active holo-nNOS dimer (8,16,17). Also, like the classic client signaling proteins, such as the GR (18), treatment of cells with geldanamycin leads to nNOS degradation via the ubiquitin-proteasome pathway (8,19). In that Hsp90 regulation of client signaling protein function reflects the ability of the chaperone to modulate ligand binding clefts (5,6), it is reasonable to predict that Hsp90 stabilization of client proteins ensues from the same cleft interaction, yet there is no evidence for this.

nNOS is a particularly useful model for exploring a relationship between Hsp90 stabilization of the protein and its interaction with the ligand binding cleft. Certain mechanism-based inactivators, such as N^G-amino-L-arginine (NAA) and the antihypertensive drug guanabenz, cause accelerated nNOS degradation (19). Guanabenz is an antihypertensive drug that produces impotence and inhibits nNOS activity, with accompanying loss of immunodetectible enzyme (20). In cultured cells, guanabenz has been shown to enhance the proteasomal degradation of nNOS (19). Guanabenz treatment leads to the oxidation of tetrahydrobiopterin and formation of a pterin-depleted nNOS that is catalytically inactive (21). The loss of tetrahydrobiopterin from its binding site within the heme/substrate binding cleft destabilizes the nNOS dimer and enhances nNOS ubiquitination (22). Many of the other inactivators cross-link heme to the enzyme (23,24), a modification that was shown in a myoglobin model to cause opening of the

heme binding cleft (25) to yield a more unfolded state of the protein (26). The reaction of the inactivator in the heme/substrate binding cleft triggers nNOS ubiquitination and proteasomal degradation (19,27).

In contrast to the stabilizing action of Hsp90, Hsp70 and its cochaperone Hsp40 are required for the degradation of many proteins (28, 29). There is considerable evidence that Hsp70 promotes degradation of proteins by promoting ubiquitination mediated by chaperone-dependent E3 ubiquitin ligases. The most studied of these is CHIP (carboxy terminus of Hsc70-interacting protein), a 35 kDa U-box E3 ubiquitin ligase (30). CHIP binds to Hsc/Hsp70 through its amino-terminal tetraticopeptide (TPR) domain (31, 32), and it binds to the UBCH5 family of E2 ubiquitin conjugating enzymes through a carboxy-terminal U-box (33). Parkin is another E3 ligase (34) that is targeted to substrate by Hsp70 (35), and for some proteins CHIP and parkin are functionally redundant in promoting degradation (4). For example, overexpression of either CHIP or parkin increases degradation of nNOS (4, 36). CHIP promotes ubiquitination of purified nNOS when it is present as the E3 ligase component in a purified ubiquitinating system, and this ubiquitination is promoted by purified Hsp70/Hsp40 (36, 37).

Most of what is known about the roles of Hsp70 and CHIP in the degradation of proteins comes from Hsp70 or CHIP overexpression experiments. To enhance mechanistic understanding of Hsp70-dependent processes in general, it would be useful to have a small molecule inhibitor of Hsp70, much as geldanamycin has been so useful in probing Hsp90-dependent effects. To this end, the Gestwicki laboratory employed a high-throughput chemical screen to identify compounds that inhibit Hsp70 ATPase activity. An inhibitor identified in the compound library was methylene blue, which was

shown to interact with purified Hsp70 by NMR spectroscopy (38). Yoshi Morishima also showed (submitted manuscript) that methylene blue inhibits the generation of steroid biding activity of the glucocorticoid receptor (GR), an established physiological action of Hsp70 (1). Activation of GR steroid binding activity by reticulocyte lysate requires Hsp70 (39), and it was shown that the methylene blue inhibition of activation is specific for the Hsp70 component of the Hsp90/Hsp70-based, multiprotein chaperone machinery.

The goal of this Chapter is to determine the effects of Hsp90 and Hsp70 on nNOS ubiquitination by a physiological ubiquitination system. The system is the DE52-retained fraction of rabbit reticulocyte lysate. This is the classic system that was originally used to resolve the components of the ubiquitin-protein ligase pathway (40). We first show that calmodulin inhibits nNOS ubiquitination in this system. However, calmodulin produces no inhibition of nNOS ubiquitination by a DE52 pool of reticulocyte lysate proteins eluted prior Hsp90. Addition of Hsp90 to this pool inhibits ubiquitination, showing it is the chaperone protein and not Ca^{2+}/calmodulin that is the direct inhibitor. We then use methylene blue to probe the role of Hsp70 in nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate. Methylene blue inhibits nNOS ubiquitination, and the block in ubiquitination is overcome by addition of purified Hsp70. Additionally, we show that nNOS ubiquitination is inhibited by anti-CHIP serum. This suggests that Hsp70-directed CHIP E3 ligase activity is responsible for nNOS ubiquitination in this system. Taken together, the data presented show that the two principal components of the Hsp90/Hsp70-based chaperone machinery have opposing actions on nNOS ubiquitination by a physiological system, with Hsp70 stimulating and Hsp90 inhibiting.

Experimental Procedures

Materials. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Protein A-Sepharose, rabbit polyclonal anti-nNOS, and purified bovine calmodulin were purchased from Sigma. GST-tagged ubiquitin and ubiquitin aldehyde were from Boston Biochem (Cambridge, MA).

Expression and Purification of nNOS, Hsp90, and Hsp70. Rat nNOS was expressed in Sf9 insect cells using a recombinant baculovirus and purified by 2',5'-ADP Sepharose and gel-filtration chromatography as described previously (8). Heme was added as an albumin conjugate during the expression to convert all of the nNOS to the holo-nNOS dimer (8). Hsp90 and Hsp70 were purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as described previously (41).

In Vitro Ubiquitination of nNOS by DE52-retained Fraction of Reticulocyte Lysate. The DE52-retained fraction of rabbit reticulocyte lysate was prepared as described previously (40). For the experiments in Fig. 2.1, purified nNOS (2 μM) was prebound with calmodulin at the indicated concentrations in the presence of 200 μM $CaCl_2$ in a total volume of 100 μl of HKD buffer (10 mM Hepes, pH 7.4, 100 mM KCl, and 5 mM DTT). An aliquot (5 μl) of this mixture was incubated for 1 h at 37 ºC with 4.5 μl of DE52-retained fraction (final concentration 7 mg protein/ml), 0.3 mg/ml bovine serum albumin, 8.3 μM GST-tagged ubiquitin, 1 mM dithiothreitol, 10 mM ATP/Mg^{2+}, 1 μl of Complete Mini protease inhibitor cocktail, 0.6 mM N-Acetyl-Leu-Leu-Nle-CHO, and 0.8 μM ubiquitin aldehyde (deubiquitination inhibitor), adjusted to a final volume of 20 μl with 1 mM Tris, pH 7.5. For the experiments in Fig. 2.3, methylene blue was

added to incubations without Ca^{2+}/calmodulin preincubation to yield the indicated final concentrations, with all samples containing a final concentration of 0.1% ethanol vehicle. Incubations were terminated by boiling with an equal volume of SDS-sample buffer containing 8 M urea and 2 M thiourea.

In Vitro Ubiquitination of nNOS by DE52 Fraction A. Prior to ubiquitination, 2 µM purified nNOS was preincubated for 15 min at 30 ºC with 4.4 µM Hsp90 and/or 30 µM of calmodulin with 0.5 mM $CaCl_2$ in a total volume of 20 µl of HKD buffer. The reaction mixture was placed on ice and diluted 2-fold with HKD buffer. An aliquot (5 µl) of this reaction mixture was added to reticulocyte lysate DE52 Fraction A (41) at a final concentration of 5.5 mg/ml, 0.3 mg/ml BSA, 0.8 µM ubiquitin-aldehyde, 0.66 mM N-Acetyl-Leu-Leu-Nle-CHO, 8.3 µM GST-tagged ubiquitin, 1 mM DTT, and 10 mM ATP. The mixtures were incubated for 1 h at 30 ºC in a total volume of 20 µl of 50 mM Tris-HCl, pH 7.5. After incubation, 20 µl of sample buffer was added and an aliquot (20 µl) was loaded for Western blotting.

Gel Electrophoresis and Western Blotting. Aliquots (10 µl) from the ubiquitination reactions were boiled in SDS sample buffer (3.75% SDS, 15% glycerol, 6 mg/ml DTT, and 0.02% bromophenol blue in 125 mM Tris-HCl, pH 6.8), resolved on 5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with anti-nNOS (1:8000). Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) and X-Omat film (Eastman Kodak Co.). The monoubiquitinated nNOS bands were scanned and the relative densities were determined with ImageJ software (http://rsb.info.nih.gov/ij/). Relative densities for 3 experiments are presented in bar graphs as percent of control or percent of the condition

with the greatest ubiquitination ± S.E. Significance of difference was determined by one-way ANOVA (Tukey's Multiple Comparison Test). Statistical probability is expressed as *p<0.05, **p<0.01, ***p<0.001.

Results

Calmodulin Inhibits nNOS Ubiquitination by the DE52-retained Fraction of Reticulocyte Lysate. We have previously reported that nNOS is ubiquitinated in vivo and that the predominant nNOS-ubiquitin conjugate detected in human embryonic kidney cells and in rat brain cytosol is the monoubiquitinated form (27). This ubiquitination was mimicked in vitro by incubating purified nNOS with an extract of rabbit reticulocyte lysate, ubiquitin and ATP (27). The extract of reticulocyte lysate consists of all of the lysate material that is retained by a DE52 column, and the DE52-retained fraction is the same as the lysate 'fraction II' that has been extensively used to study protein ubiquitination (40). The DE52-retained fraction contains Hsp90, Hsp70 and Hsp40, as well as the ubiquitinating enzymes, with all the components being present in the same ratios as exist in reticulocyte lysate (8,27).

Because of the mutual interaction between calmodulin and Hsp90 reported for the NOS enzymes (11,12,14,15), we asked whether CaM would affect nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate. As shown in Fig. 2.1, incubation of purified nNOS with the DE52-retained fraction and GST-ubiquitin yields a slower migrating band (lane 2) that has been previously demonstrated to be the major

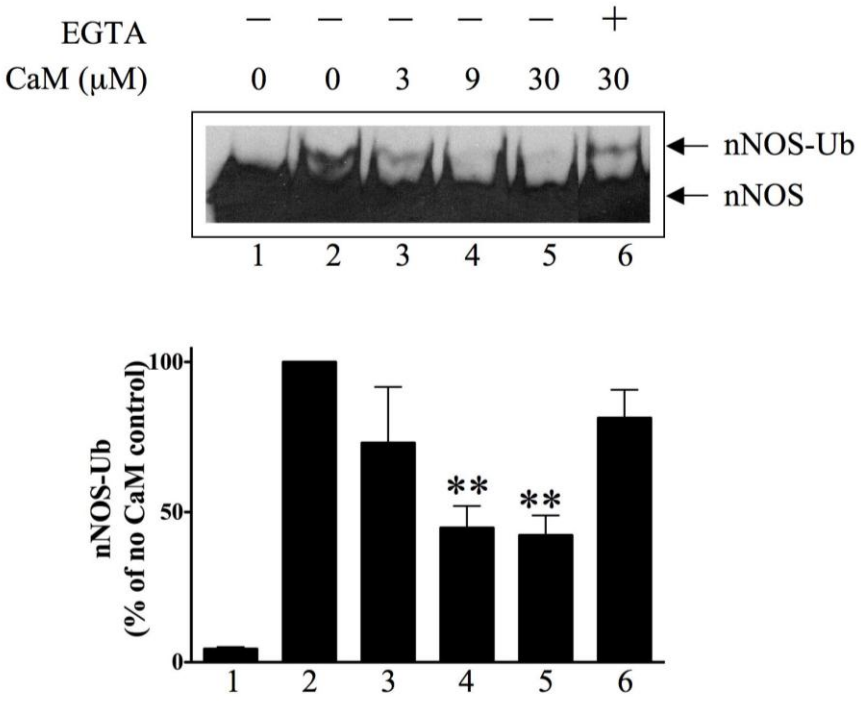

Figure 2.1. Calmodulin inhibition of nNOS ubiquitination by the DE52-retained fraction of rabbit reticulocyte lysate. nNOS monoubiquitin conjugates (nNOS-Ub) were detected by Western blot. nNOS was incubated for 1 h at 37 °C with a DE52-reatined fraction of rabbit reticulocyte lysate, ATP, GST-ubiquitin and the indicated concentrations of calmodulin as described under Methods. Samples were Western blotted by probing with anti-nNOS. Lane 1, incubation time 0; lanes 2-6, incubation time 1 h. For bar graph, the relative amounts of nNOS-Ub in replicate experiments was determined by scanning and expressed as % of the 1 h control without calmodulin. The values are the mean ± S.E. (n=3). ** denotes significantly (p<0.01) lower than nNOS-Ub conjugates relative to the 1 h control without CaM.

monoubiquitinated nNOS product (27). Addition of calmodulin inhibits nNOS ubiquitination (lanes 3, 4, and 5) in a calcium-dependent manner, as indicated by the increased ubiquitination seen in the presence of EGTA (lane 6). The amount of ubiquitinated product relative to the control without added calmodulin is presented for several experiments in the bar graph of Fig. 2.1. It should be noted that because only a small fraction of nNOS is ubiquitinated, the assay contains a lot of the purified enzyme to serve as substrate, as is indicated by the broad bands of unmodified nNOS in Fig. 2.1. Given the large amount of nNOS, we have tested the effect of CaM over a range of concentrations that reflect a reasonable stoichiometry with the enzyme. To give a point of reference, the ratio of CaM to nNOS in our enzyme activity assay is 11:1 and the highest concentration of CaM (30 μM) in the ubiquitination mixture yields a ratio of 15:1.

Calmodulin Acts by Potentiating Inhibition of Ubiquitination by Hsp90. The calmodulin inhibition of ubiquitination by Hsp90 shown in Fig. 2.1 could be due to calmodulin itself or to calmodulin potentiation of ubiquitination inhibition produced by Hsp90. To examine this, Hwei-Ming Peng added calmodulin, purified Hsp90 or both to incubations containing purified nNOS and purified E1, E2, and E3 ubiquitinating enzymes. Because CHIP was used as the E3 ubiquitin ligase, nNOS was preincubated with purified Hsp70/Hsp40 and ATP prior to ubiquitination. In this purified system, calmodulin alone did not inhibit ubiquitination, Hsp90 alone inhibited ubiquitination, and calmodulin potentiated inhibition by Hsp90 (37).

These results with the purified ubiquitination system suggest that the inhibition of nNOS ubiquitination observed when calmodulin is added to the DE52 fraction of reticulocyte lysate in Fig. 2.1 is caused by Hsp90. To demonstrate this, we prepared an

Hsp90-free pool of reticulocyte lysate fractions eluted from DE52 with a gradient of KCl, as was described in Dittmar *et al.* (41). The pooled fractions that elute before Hsp90 contain Hsp70, Hsp40, and nNOS ubiquitinating activity. We have called this Hsp90-free pool Fraction A (41). As shown in Fig. 2.2, addition of calmodulin to Fraction A increases nNOS ubiquitination (cf. lanes 2 and 3) (probably because nNOS activation and formation of reactive oxygen species), whereas addition of Hsp90 inhibits ubiquitination (lanes 4 and 5).

Use of Methylene Blue as a Tool to Determine Hsp70 Dependence of nNOS Ubiquitination. Because a good Hsp70 inhibitor has not been available, it has been difficult to establish whether or not ubiquitination events are Hsp70-dependent. It is known, for example, that the reaction of certain inactivators in the heme/substrate binding cleft of nNOS triggers its ubiquitination and degradation (19, 27) and that overexpression of either CHIP or parkin promotes nNOS degradation (4, 36). These data suggest that Hsp70 may be involved. Ubiquitination of purified nNOS by a purified system using CHIP as the E3 ligase is promoted by purified Hsp70 (36, 37), but it is not known if nNOS ubiquitination by a physiological ubiquitinating system is Hsp70-dependent. Thus, we used the DE52-retained fraction of reticulocyte lysate to test the effectiveness of methylene blue in detecting the Hsp70 dependence of nNOS ubiquitination.

To determine if ubiquitination by this system requires Hsp70, nNOS was incubated with the DE52-retained fraction in the presence of increasing concentrations of methylene blue. As shown in Fig. 2.3, methylene blue inhibits nNOS ubiquitination. The concentration of Hsp70 in this ubiquitinating system is ~5% of Hsp70 in whole

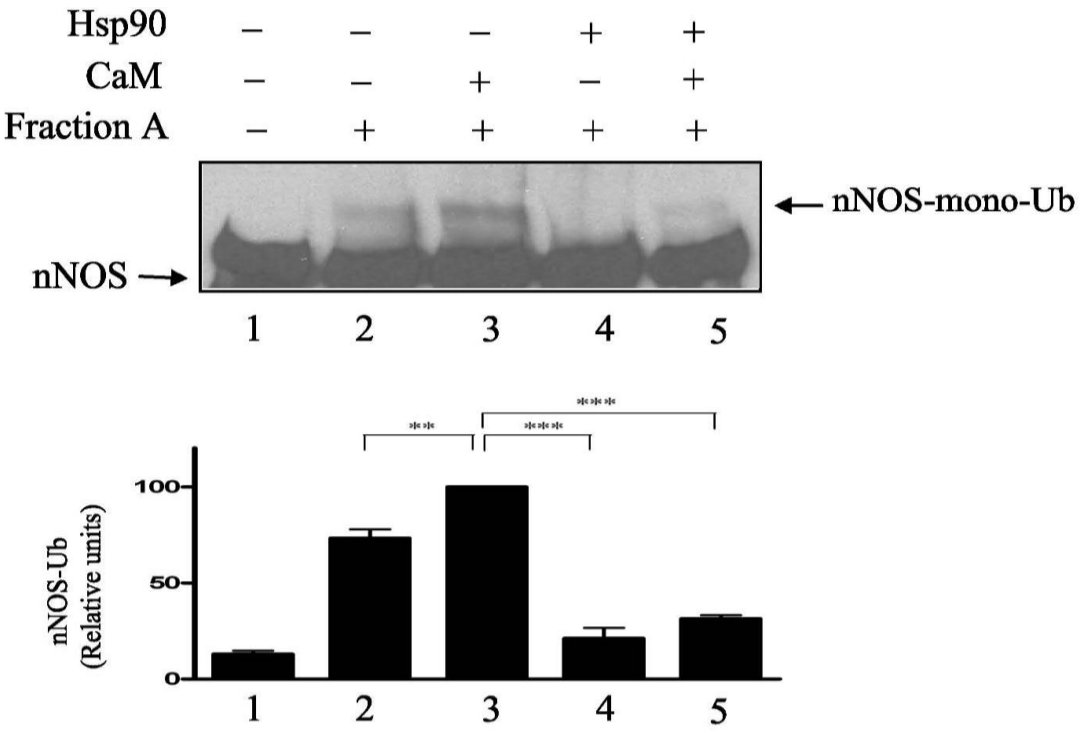

Figure 2.2. Calmodulin does not inhibit and Hsp90 does inhibit nNOS ubiquitination by Fraction A of reticulocyte lysate. nNOS was preincubated with 30 μM CaM and/or Hsp90 as indicated and then incubated with Hsp90-free Fraction A of reticulocyte lysate. The samples were Western blotted by probing with anti-nNOS.

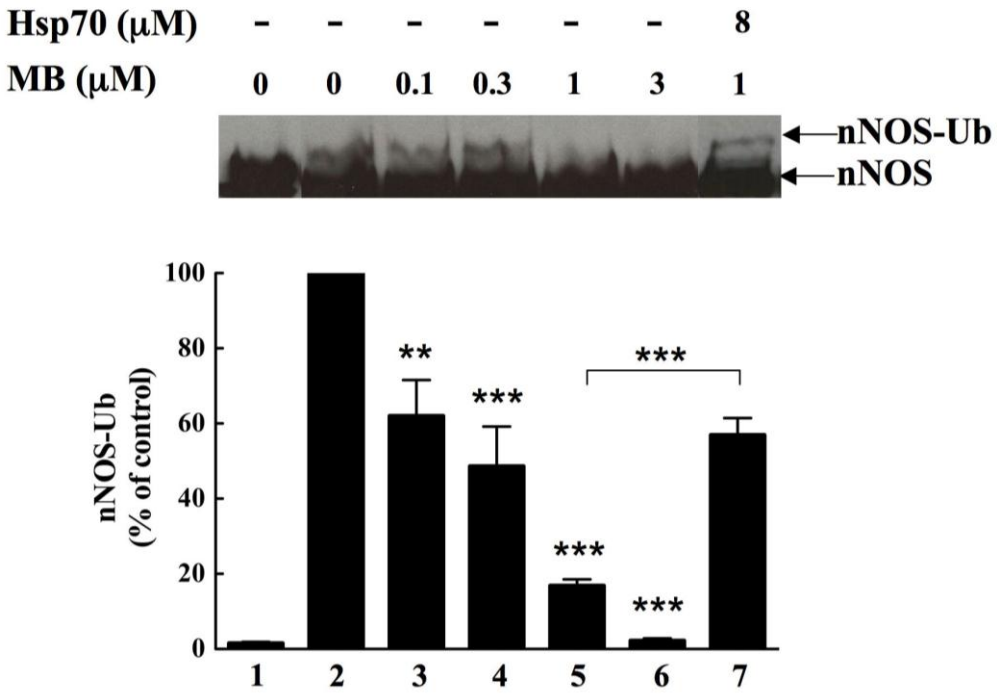

Figure 2.3. Methylene blue inhibits nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate in an Hsp70-dependent manner. Purified nNOS was incubated 1 h at 37 °C with the DE52-retained fraction of reticulocyte lysate, ATP, GST-ubiquitin, and the indicated concentrations of methylene blue. In addition 8 μM purified Hsp70 was added to a sample of the DE52-retained fraction containing 1 μM methylene blue. Samples were Western blotted by probing with anti-nNOS. Lane 1, incubation time 0; lanes 2-7, incubation time 1 h. For bar graph, the relative amount of monoubiquitinated nNOS (nNOS-Ub) in replicate experiments was determined by scanning and expressed as % of the one hour control without methylene blue. The values are the mean ± S.E. (*n*=3). Asterisks over the columns denote significantly different from control and asterisks over the line denote that condition 7 is significantly different from condition 5.

reticulocyte lysate and much lower concentrations of methylene blue are effective in inhibiting ubiquitination. Importantly, the inhibition produced by 1 μM methylene blue is largely overcome when purified Hsp70 is added to the incubation mix (Fig. 2.3, lane 7). This shows that nNOS ubiquitination in a physiological system is Hsp70-dependent. This also suggests that methylene blue may be a useful reagent to detect Hsp70-dependent effects, much as geldanamycin has been useful to probe for Hsp90-dependent effects.

nNOS Ubiquitination by the DE52-retained Fraction is CHIP-dependent. Although overexpression of CHIP promotes nNOS degradation (36) and CHIP directs nNOS ubiquitination in a purified ubiquitination system (36, 37), it is not known if CHIP is the dominant E3 ligase for nNOS ubiquitination by a physiological ubiquitination system. To assess this, purified nNOS was incubated with the DE52-retained fraction of reticulocyte lysate in the presence of anti-CHIP antibody. As shown in Fig. 2.4, nNOS ubiquitination is markedly reduced in the presence of anti-CHIP serum (lane 4) compared to nonimmune serum (lane 3). Taken together, the data of Figs. 2.3 and 2.4 suggest that nNOS ubiquitination by this model physiological ubiquitinating system is both Hsp70-dependent and CHIP-dependent, with the ubiquitinating activity being inhibited by methylene blue.

Discussion

It is clear from Fig. 2.1 that nNOS ubiquitination by the reticulocyte lysate system is inhibited by Ca^{2+}/calmodulin. However, addition of CaM alone to a purified CHIP-dependent ubiquitination system did not yield inhibition of ubiquitination, whereas

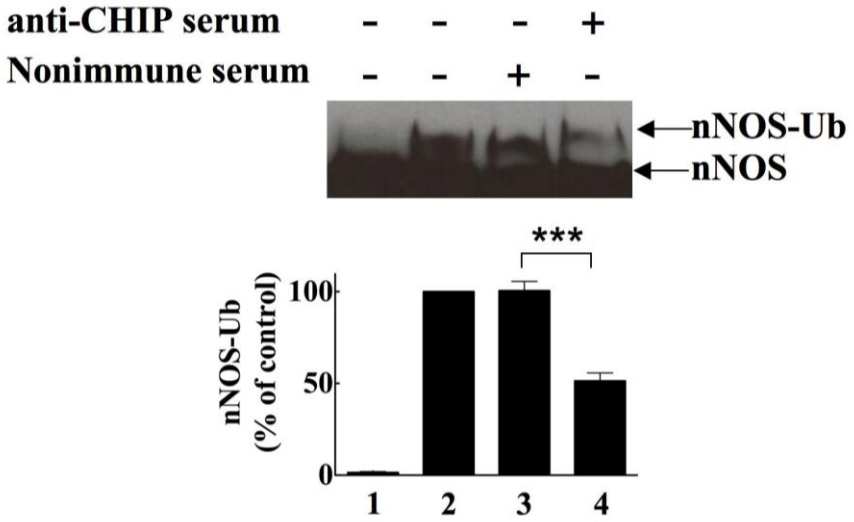

Figure 2.4. CHIP is the major E3 ligase for nNOS ubiquitination by the DE52-retained fraction of rabbit reticulocyte lysate. Purified nNOS was incubated with the DE52-retained fraction of reticulocyte lysate as above, but in the presence of 1% nonimmune serum or 1% anti-CHIP serum. Lane 1, incubation time 0; lanes 2-4, incubation time 1 h.

Hsp90 inhibited ubiquitination by the purified system when added alone and the simultaneous presence of CaM increased this inhibition (37). This suggests that the inhibition of nNOS ubiquitination observed in the reticulocyte lysate system when CaM is added could be due to Hsp90. When nNOS is ubiquitinated by an Hsp90-free fraction of reticulocyte lysate, addition of CaM does not inhibit nNOS ubiquitination but addition of purified Hsp90 does inhibit (Fig. 2.2). The fact that CaM interaction with nNOS enhances both Hsp90 stimulation of nNOS activity (14, 15) and Hsp90 inhibition of nNOS ubiquitination is consistent with a model in which the two effects of Hsp90 are caused by the same interaction of the chaperone with the enzyme.

It is established that Hsp90 binds to the oxygenase domain of eNOS (42). This domain contains the heme/substrate binding cleft, and it is likely the site of Hsp90 interaction with nNOS as well. Inasmuch as CaM enhances electron flux from flavin bound to the reductase domain to the heme bound within the cleft (43), CaM binding is likely to affect the state of the cleft. Although it is unclear exactly how CaM affects cleft structure/mobility, it is clear that CaM and Hsp90 affect both eNOS and nNOS in such a manner as to increase the binding of the other (11, 12, 14, 15). It has been proposed that Hsp90 interacts directly with the heme/substrate binding cleft when it inhibits ubiquitination (6, 37). It is this ability to inhibit ubiquitination that likely accounts for Hsp90 stabilization of a wide variety of proteins to proteasomal degradation. Pratt *et al.* (5, 6) have previously proposed that Hsp90 acts to stabilize an open state of the ligand binding cleft, an effect that both facilitates substrate access to increase enzyme activity and prevents further cleft unfolding that triggers Hsp70-dependent ubiquitination.

Several laboratories have been interested in developing small molecule inhibitors of Hsp70 for potential use in the treatment of cancers as well as neurodegenerative diseases characterized by the accumulation of aberrant proteins (44-49). Methylene blue was identified in a screen for compounds that inhibit Hsp70 ATPase activity (38). Unlike geldanamycin, which binds in the unique nucleotide binding pocket of Hsp90 and produces effects that are quite specific for inhibition of Hsp90 family proteins in eukaryotes (1), methylene blue has multiple cellular and molecular targets, including multiple neurotransmitter systems, ion channels and enzymes (reviewed in Ref. 50). Although modulation of cGMP signaling is often considered its most significant effect, the redox properties of methylene blue are utilized in the treatment of methemoglobinemias and ifosfamide-induced encephalopathy, and probably account for its use as an antimicrobial agent (50). Given its multiple molecular targets, methylene blue would seem *a priori* to be an imprecise research tool for probing Hsp70-dependent effects. Yet, we indicate here that this readily available compound can be used for this purpose.

Overexpression of CHIP has been shown to promote proteasomal degradation of a wide variety of normal and aberrant proteins. Although overexpression of CHIP promotes the proteasomal degradation of nNOS (36), there is clear redundancy in E3 ligase action on nNOS (4), and overexpression of one E3 ligase could favor a normally minor pathway of ubiquitination. Thus, it was not previously established that Hsp70-dependent CHIP activity is the principle physiologic pathway for nNOS ubiquitination. Methylene blue causes virtually complete inhibition of nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate (Fig. 2.3). This finding suggests that all of

the nNOS ubiquitination by the reticulocyte system may be Hsp70-dependent. Similarly, inhibition by anti-CHIP antibody (Fig. 2.4) suggests that CHIP is a major E3 ligase for nNOS in reticulocytes. Even though methylene blue affects a variety of biochemical processes, including a well established inhibition of the NOS enzymes (51, 52), these data demonstrate that it can be used as a research tool to identify Hsp70-mediated processes in cell-free systems.

It has not been known how proteins that have undergone oxidative or toxic damage are recognized and shunted to the ubiquitin-proteasome pathway of degradation. A model of nNOS triage is presented in Fig. 2.5. The effects of guanabenz and NAA serve as examples of such toxic damage that is targeted to the ligand binding cleft and triggers ubquitination of nNOS. Other examples of mechanism-based protein damage triggering ubiquitination and proteasomal degradation have been discussed in a review (5). It is reasonable to propose that, as proteins undergo such toxic damage, ligand binding clefts open, exposing hydrophobic residues as the initial step in unfolding. As long as Hsp90 can form even transient complexes with the opening cleft, ubiquitination by Hsp70-dependent ubiquitin E3 ligases, like CHIP, is inhibited. But a point is reached where unfolding of the cleft progresses to a state that cannot cycle with Hsp90, and ubiquitination directed by Hsp70-dependent E3 ligases is unopposed. In this way, the Hsp70/Hsp90 chaperone machinery may be the major mechanism by which the quality control decision is made for degradation of damaged proteins via the ubiquitin-proteasome pathway.

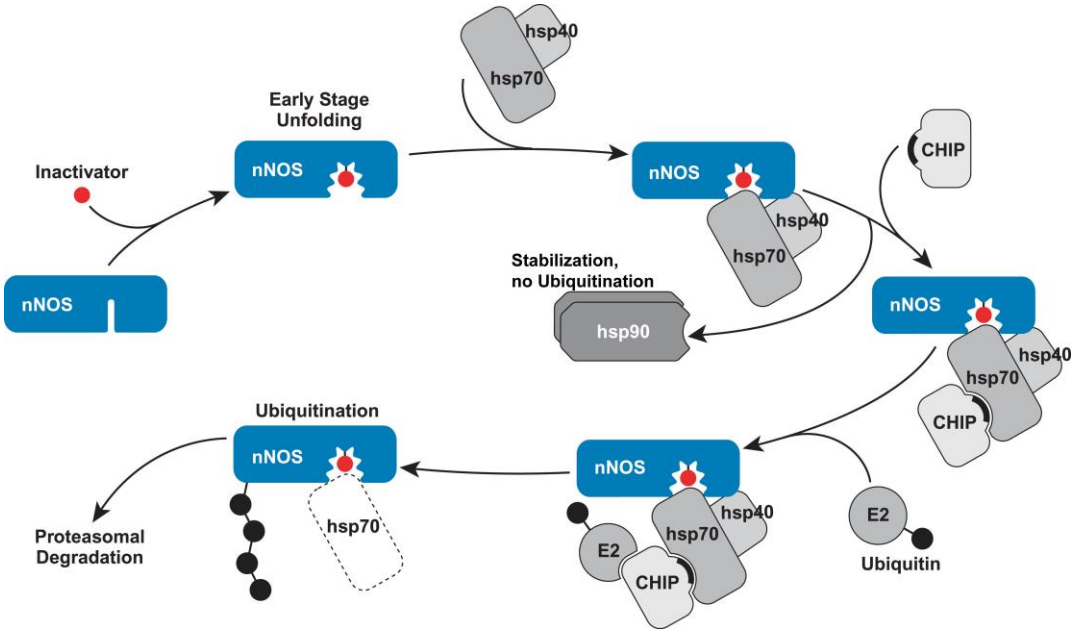

Figure 2.5. Proposed model for triage of nNOS by the Hsp90/Hsp70-based chaperone machinery after mechanism-based inactivation. Mechanism-based inactivation of nNOS by certain substrates leads to unfolding of the heme/substrate binding cleft to a degree that Hsp90 cannot cycle with the enzyme to inhibit ubiquitination by Hsp70-dependent E3 ligases, such as CHIP. The *solid crescent* represents the CHIP TPR domain.

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CHAPTER 3

THE C331A MUTANT OF NEURONAL NITRIC-OXIDE SYNTHASE IS LABILIZED FOR HSP70/CHIP (C-TERMINUS OF HSC70-INTERACTING PROTEIN)-DEPENDENT UBIQUITINATION

Summary

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 recticulocyte 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, whereas 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.

Introduction

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: neuronal NOS (nNOS), 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). CHIP (carboxyl terminus of the Hsc70-interacting protein) 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 nNOS 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 crosslinking 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 CXXXXC motif that provide the thiolate ligation for a tetradentate zinc binding site (17). The zinc is a structural and not a catalytic feature of nNOS. The C331A nNOS mutant was first described by Dr. Masters' group and found to affect tetrahydrobiopterin and L-arginine binding (18). 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 synthesize 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 N^G-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 N^G-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 previously described 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-biopterin (BH4) was purchased from Dr. Schirck's Laboratory (Jona, Switzerland). Protein A-Sepharose, ubiquitin, ATP, creatine phosphokinase, L-arginine, N^G-nitro-L-arginine, N^G-nitro-D-arginine, A23187, and rabbit polyclonal anti-nNOS were purchased from Sigma (St. Louis, MO). HRP-tagged goat anti-rabbit secondary antibody was from Chemicon (Temecula, CA). Rabbit polyclonal anti-CHIP antibody and N-Acetyl-Leu-Leu-Nle-CHO was from Calbiochem (Gibbstown, NJ). 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 phosphate was from Fluka (St. Louis, MO). Complete Mini protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN). Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was from QIAGEN Inc (Valencia, CA). The cDNA for rat neuronal NOS was kindly provided by Dr. Solomon Snyder (Johns Hopkins Medical School, Baltimore, MD). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (Univ. of Michigan). The cDNA for expressing the UbcH5a−GST fusion protein was kindly provided by C. M. Pickart (Johns Hopkins Medical School, Baltimore, MD). pET30aCHIP plasmid for expressing His-CHIP was kindly provided by C. Patterson (University of North Carolina, Chapel Hill, NC).

Expression and Purification of nNOS, C331A nNOS, Hsp70, Hsp40, 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 (19), except that BL21 E. 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 (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 Ni-NTA affinity chromatography as previously described (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 (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 mM 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 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 oC 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 Hepes, pH 7.5. The reaction mixture was placed on ice and diluted 2-fold with 10 mM Hepes buffer, pH 7.4, containing 100 mM KCl, and 5 mM DTT. To conjugate Ub 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 oC 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.

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 oC 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 Hepes, pH 7.5. The reaction mixture was placed on ice and diluted 2-fold with 10 mM Hepes buffer, pH 7.4, containing 100 mM KCl, and 5 mM DTT. To conjugate Ub 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 oC 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 previously described (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 (Univ. 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-Polyacrylamide Gel Electrophoresis, 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 HEPES, pH 7.4, 0.32 M sucrose, 2 mM EDTA, 6 mM PMSF, 10 mg/ml leupeptin, 2 mg/ml aprotinin, 10 mg/ml trypsin inhibitor, 10 mM sodium vanadate, 1% (w/v) NP-40, and 5 mM N-ethylmaleimide). Homogenates were centrifuged for 30 min at 14,000 x g, and the supernatant was taken for immunoprecipitation of nNOS. The nNOS was immunoadsorbed 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 oC. 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) bromophenol 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 volts. The blot was probed with anti-nNOS (0.01%, w/v) and then an HRP-tagged goat anti-rabbit secondary antibody (0.0025%, w/v) and the immunoreactive bands were 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 (NIH).

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 (28). In this procedure, nitrate reductase (Boehringer Mannheim) was used to convert the nitrate to nitrite, which was quantified by the use of the Griess regent. 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 to 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. 3.1A, the major ubiquitin conjugate of the C331A nNOS is also the monoubiquinated 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. 3.1B, 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. 3.1C, the L-, but not D-, isomer of N^G-nitroarginine decreases the ubiquitination of C331A nNOS.

As shown in Fig. 3.2A, the omission of tetrahydrobiopterin from the incubation mixture has no effect on the ubiquitination of the C331A nNOS (open bars, cf. condition 1 with condition 2) but greatly increases the ubiquitination of the wild type nNOS (solid bars, cf. condition 3 with 4). The labilized wild type nNOS is protected from ubiquitination by L-, but not D-, N^G-nitroarginine (solid bar, cf. condition 5 with condition 6). In Fig. 3.2B, the concentration dependence of N^G-nitro-L-arginine for protection against ubiquitination is presented for both the wild type (squares) and the C331A mutant of nNOS (triangles). The N^G-nitro-L-arginine protects the wild type nNOS and the C331A nNOS with an IC50 of 0.2 ± 0.3 μM and 8.7 ± 0.3 μM,

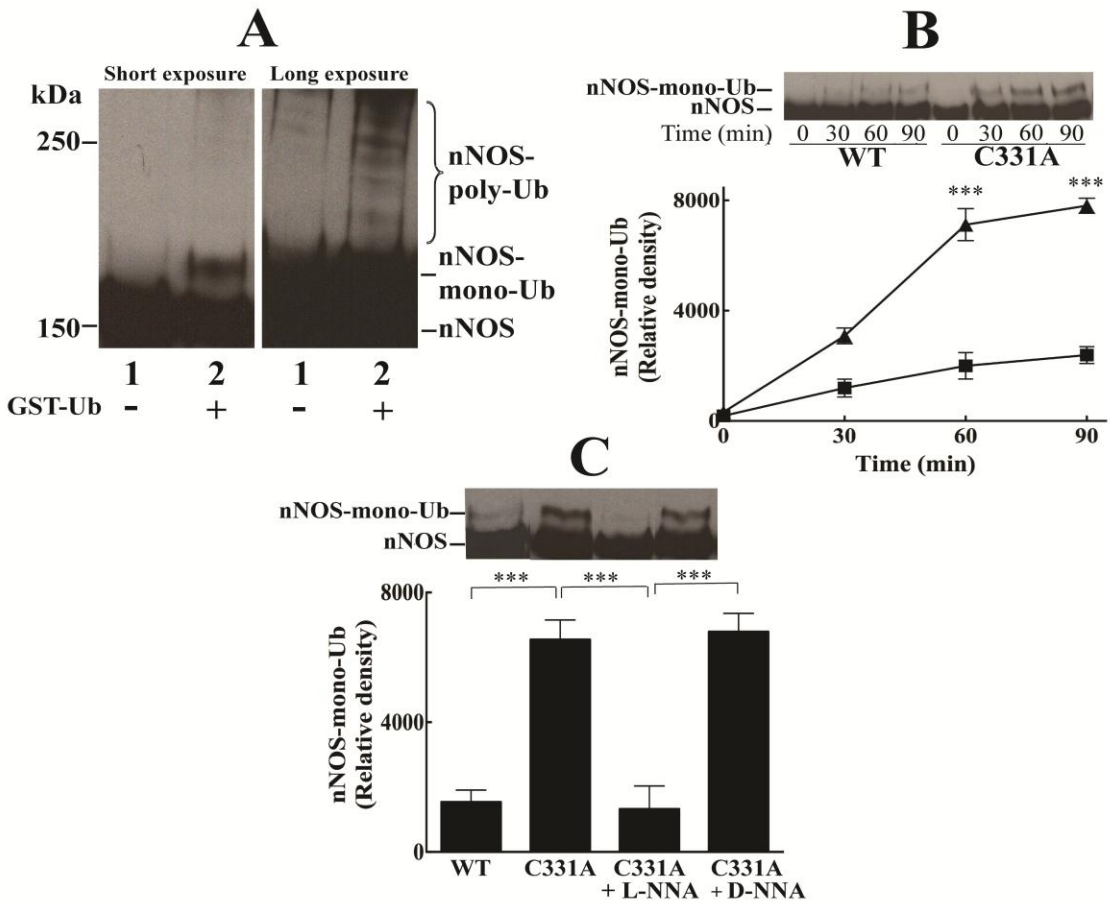

Fig. 3.1. C331A nNOS mutant is preferentially ubiquitinated in Fraction II by a process that is attenuated by N^G-nitro-L-arginine, but not N^G-nitro-D-arginine. The ubiquitination of purified wild type and C331A mutant of nNOS by Fraction II was performed as described in *Experimental Procedures*. *A*, 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-dependant formation of a higher molecular mass band that corresponds to the mono-ubiquitinated nNOS (nNOS-Ub). A representative blot is shown in the upper panel. The nNOS-Ub was quantified by the use of ImageJ software (NIH) and plotted in the lower panel. Triangles, C331A nNOS ubiquitin conjugates; squares, wild type nNOS ubiquitin conjugates. The values are the mean ± S.E. (n=3). *C*, the effect of N^G-nitro-L-arginine (L-NNA) or N^G-nitro-D-arginine (D-NNA) on the ubiquitination of C331A mutant of nNOS (C331A). The N^G-nitro-arginine compound was added to the ubiquitination mixture to give a final concentration of 25 μM and the amount of nNOS-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 ***p<0.001.

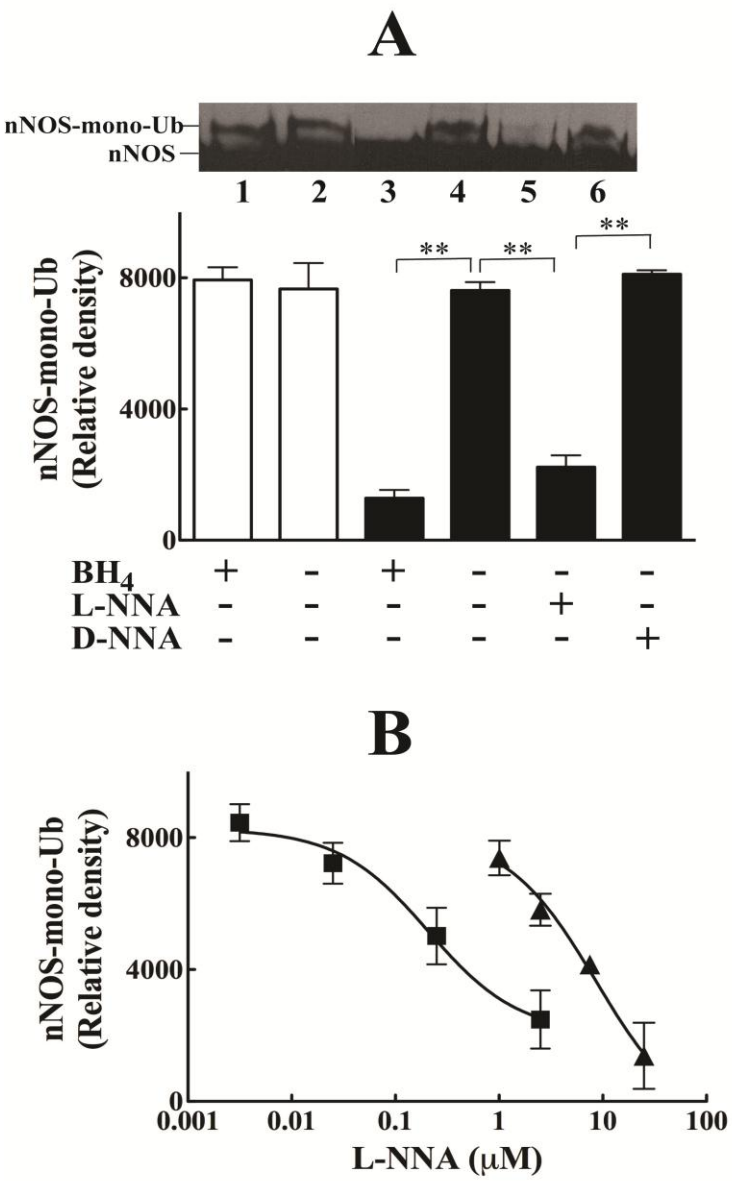

Fig. 3.2. Omission of tetrahydrobiopterin enhances the ubiquitination of wild type nNOS to the level seen for the C331A mutant of nNOS. The ubiquitination of nNOS catalyzed by Fraction II was performed as described in *Experimental Procedures. A*, the amount of ubiquitin conjugated to C331A mutant of nNOS (open bars) or to that of the wild type nNOS (solid bars) was measured after incubation for 1h at 37 °C. As indicated, tetrahydrobiopterin (BH_4) was omitted in certain samples and N^G-nitro-L-arginine (L-NNA) or N^G-nitro-D-arginine (D-NNA) were added in certain samples to give a final concentration of 2.5 μM. *B*, the dependence on the concentration of N^G-nitro-L-arginine (L-NNA) on the ubiquitination of wild type (squares) and C331A mutant (triangles) of nNOS. Tetrahydrobiopterin was omitted from the incubation mixtures. The values in both panels represent the mean ± S.E. (n=3). Statistical probability is expressed as $^{**}p<0.01$.

respectively. The greater than 40-fold difference in IC50 values for N^G-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.

The 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. 3.3A, 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 (lane 5) and this inhibition is substantially blunted by the addition of purified Hsp70 (lane 7). Thus, C331A nNOS ubiquitination is dependent on Hsp70. As indicated earlier, the changes measured in the monoubiquitin conjugate reflect the changes seen in the polyubiquitin conjugates. As shown in Fig. 3.3B, 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 ($r^2 = 0.84$) between the mono and polyubiquitin conjugates formed. As shown in Fig 3.3C, 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 (lane 5). This suggests that CHIP is the major ubiquitin ligase for C331A nNOS in fraction II. As a

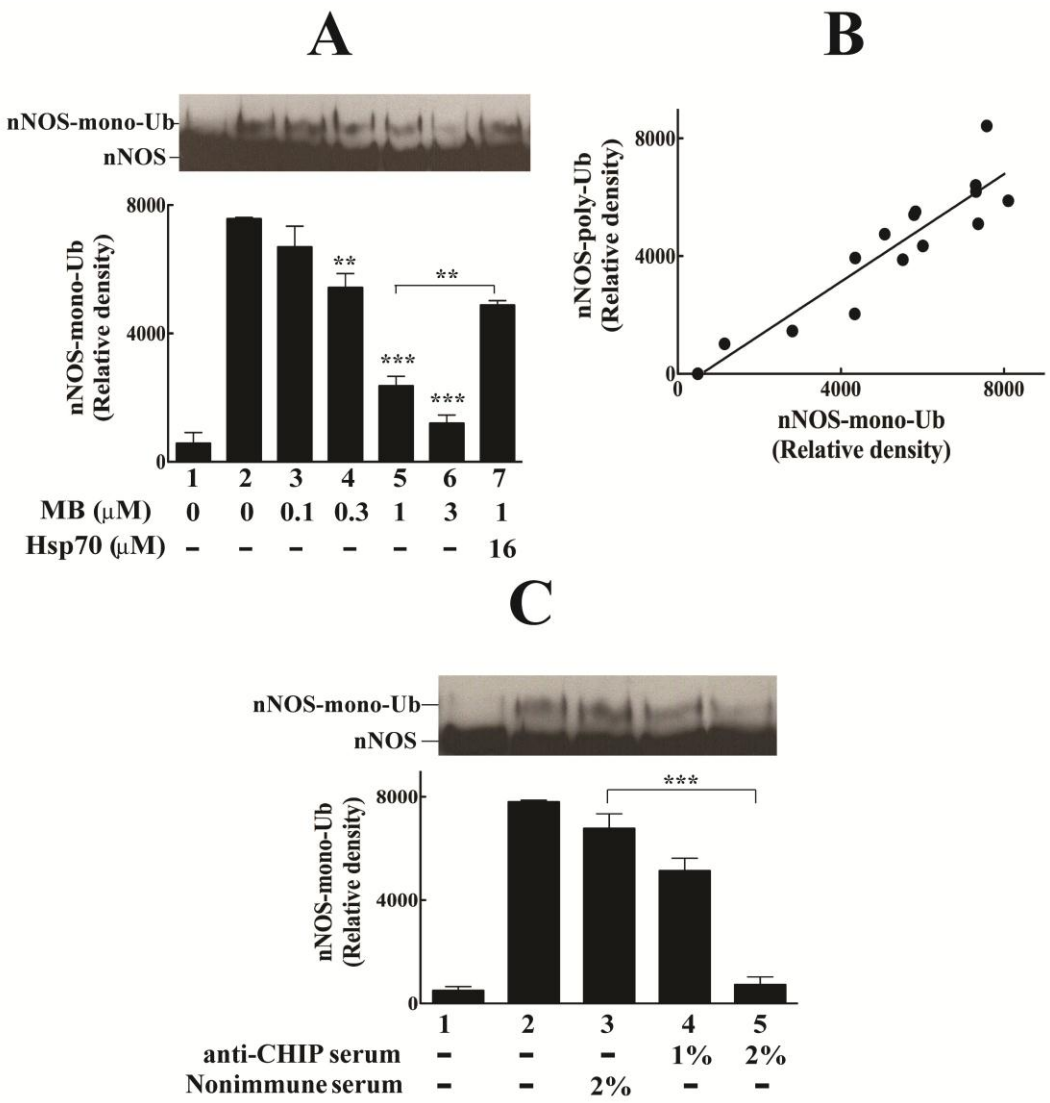

Fig. 3.3. Hsp70 and CHIP dependence of the ubiquitination of C331A nNOS catalyzed by Fraction II. The ubiquitination of C331A nNOS catalyzed by Fraction II was performed as described in *Experimental Procedures*, except that the indicated concentrations of methylene blue (MB) were added. Samples were Western blotted by probing with anti-nNOS and an HRP-tagged goat anti-rabbit secondary antibody. *A*, the relative amounts of nNOS-Ub from replicate experiments was determined by scanning and 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*, the amount of mono-ubiquitin conjugate in each sample in A was plotted against the amount of poly-ubiquitin conjugates. The relative amount of polyubiquitin conjugate was determined by scanning the area depicted in Fig. 3.1A. A line was fit to the data with an r^2 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% nonimmune serum or 1% or 2% anti-CHIP serum was added as indicated. Lane 1, incubation time 0; lanes 2-4, incubation time 1 h. Statistical probability is expressed as $^{**}p<0.01$, $^{***}p<0.001$.

control, we found that 2% non-immune serum has no significant effect on the ubiquitination (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 required components of a ligase system, including purified ubiquitin activating enzyme (E1) and an ubiquitin conjugating enzyme (E2) (31-33). CHIP was shown to be an 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 if CHIP acts as an ubiquitin ligase for C331A nNOS. As shown in Fig. 3.4A, 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 bracket were quantified and plotted in Fig. 3.4B. The C331A nNOS is ubiquitinated to a greater degree than the wild type enzyme (cf. condition 1 with condition 2). Moreover, the L-, but not D-, N^G-nitroarginine protects C331A nNOS from ubiquitination by the purified protein system (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. 3.4B, 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 an ubiquitin ligase for C331A nNOS. The omission of added Hsp70 (condition 7) decreased ubiquitination by approximately 40%, consistent with previous

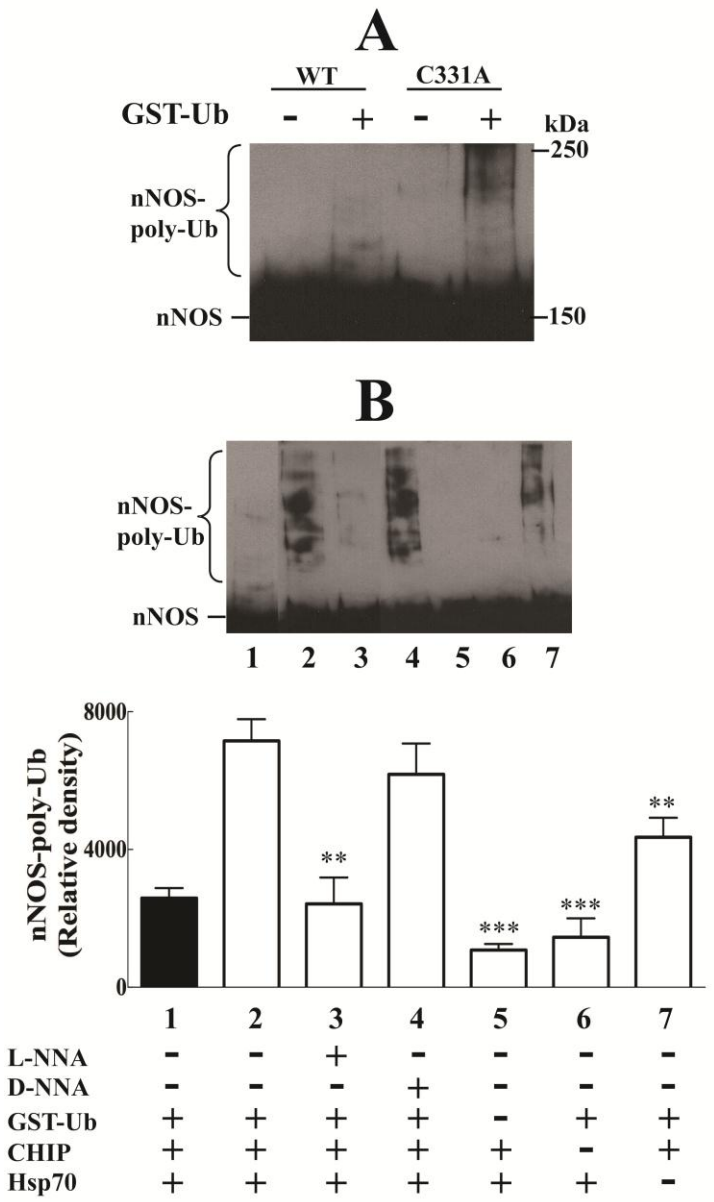

Fig. 3.4. C331A nNOS is preferentially ubiquitinated in an in vitro system containing purified Hsp70/Hsp40, E1, E2, and CHIP. The ubiquitination of nNOS catalyzed by the purified ubiquitination system was performed as described in *Experimental Procedures. A*, immunoblot showing the ubiquitin conjugate of wild type nNOS (WT) or C331A mutant of nNOS (C331A). *B*, the ubiquitin conjugate of wild type nNOS (lane 1) or C331A mutant of nNOS (lane 2) was quantified and plotted. The effects of N^G-nitro-L-arginine (L-NNA) or N^G-nitro-D-arginine (D-NNA) on the ubiquitination of C331A mutant of nNOS are also shown (lane 3 and lane 4). The effect of omission of GST-tagged ubiquitin (GST-Ub, lane 5), CHIP (lane 6), or Hsp70 (lane 7) are also shown. Statistical probability is expressed as $^{**}p<0.01$, $^{***}p<0.001$.

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 Km and Vmax 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 Km and Vmax 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 the effect of L-arginine on stability and ubiquitination. As shown in Fig. 3.5A, 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 (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 (solid circles). D-arginine at the same concentration does not stabilize the C331A nNOS (solid triangles). As shown in Fig. 3.5B, 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. 3.4B. The D-arginine did not affect the ubiquitination of C331A nNOS (Fig. 3.5B). Thus, the

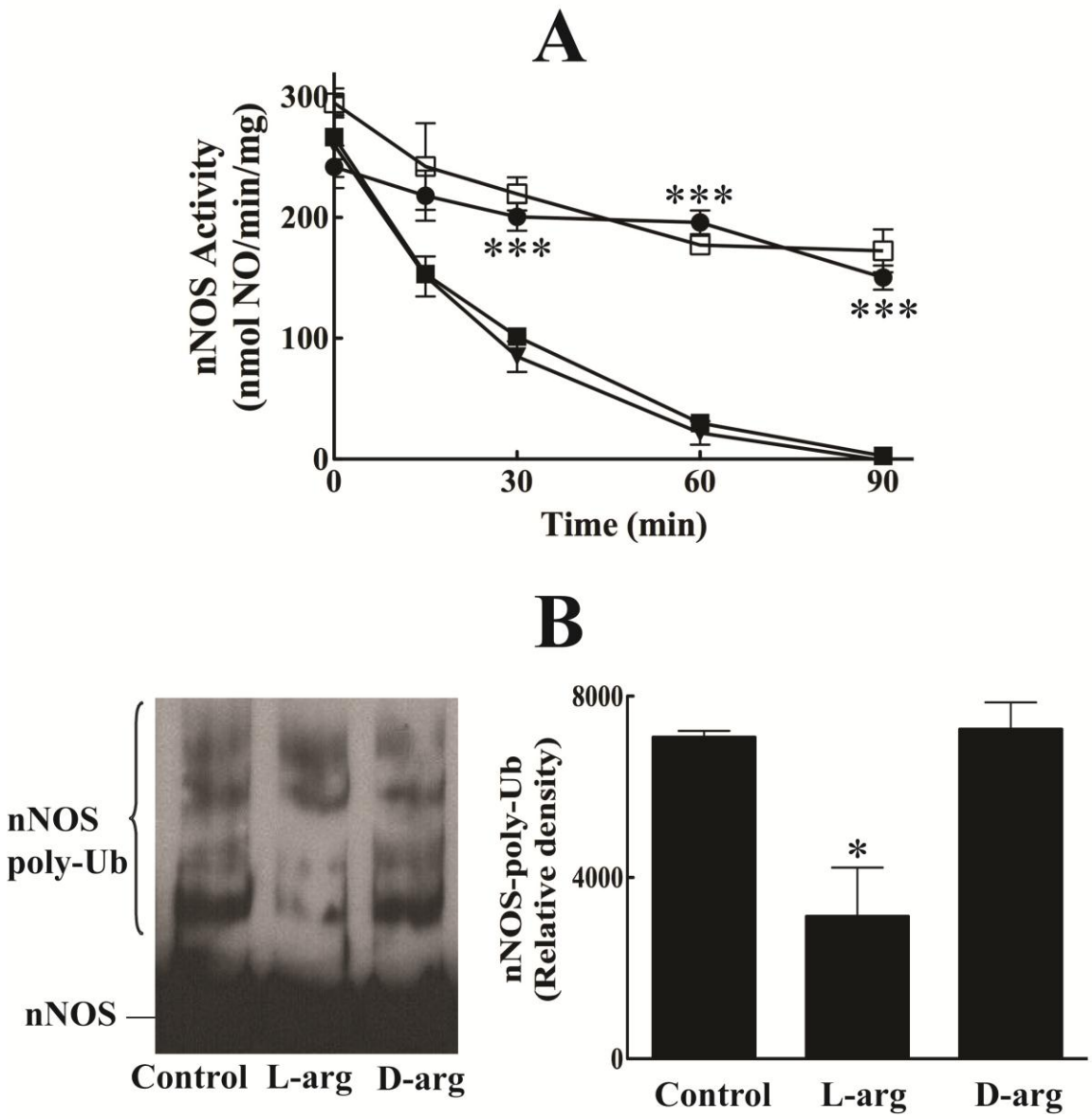

Fig. 3.5. C331A nNOS is destabilized and becomes a substrate for ubiquitination. *A*, the catalytic stability of wild type nNOS and C331A nNOS was examined. Purified C331A nNOS (2 μM) was incubated at 30 °C with 10 μM BH_4 in a total volume of 30 μl of 250 mM Tris, pH 7.5. In some cases, 100 μM L-arginine 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 in *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*, the 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. 3.4. Statistical probability is expressed as *p<0.05, ***p<0.001.

inactive form of C331A nNOS, which was initially described by Dr. Masters' lab (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 if 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 (25). The fraction pool that elutes before Hsp90 contains Hsp70, Hsp40 and nNOS ubiquitinating activity and we call it fraction A (25). As shown in Fig. 3.6A, fraction A ubiquitinates C331A nNOS and the addition of purified Hsp90 decreases C331A nNOS ubiquitination in a concentration-dependent manner. It is noteworthy that the amount of Hsp90 in reticulocyte lysate (or fraction II) has been determined to be 4 to 5 μM (34). The inhibition of ubiquitination of C331A nNOS by Hsp90 was similar to that found for the wild type nNOS, with nearly complete inhibition at 13.2 μM. As described earlier, the C331A nNOS is unstable and the enzyme activity decreases over time. As shown in Fig. 3.6B, 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.

Ubiquitination of C331A nNOS in HEK293T Cells and the Effect of N^G-nitroarginine. We wished to compare the ubiquitination of wild type nNOS to that of C331A nNOS in intact cells. As shown in Fig. 3.7A, transient transfection of wild type nNOS or C331A nNOS and subsequent immunopurification of the overexpressed enzyme after treatment with MG132, a proteasome inhibitor, gave one major product that was previously

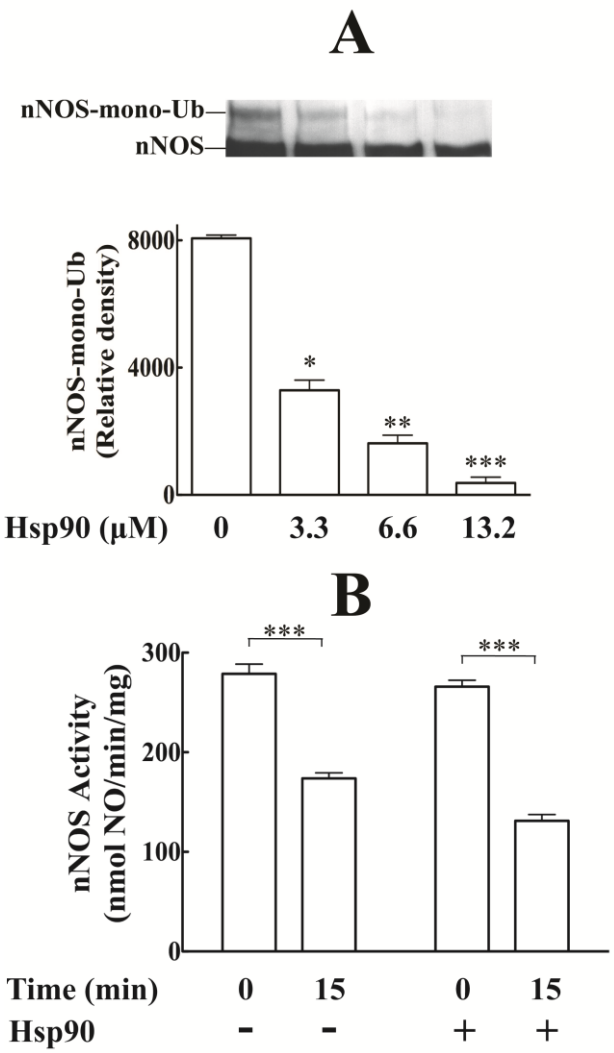

Figure 3.6. Hsp90 blocks ubiquitination, but does not stabilize activity, of C331A nNOS. A, the ubiquitination of C331A nNOS catalyzed by Fraction A was performed as described in *Experimental Procedures*, except that the indicated concentrations of purified Hsp90 were added. Samples were Western blotted by probing with anti-nNOS and an HRP-tagged goat anti-rabbit secondary antibody. The relative amounts of nNOS-Ub from replicate experiments were determined by scanning and expressed as relative density and plotted. The values are the mean ± S.E. (n=3). **B,** Purifed C331A nNOS (2 μM) was incubated at 30 °C with 10 μM BH_4, 500 μM $CaCl_2$, 30 μM calmodulin, 2 μl of an ATP-generating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase), in a total volume of 20 μl Tris, pH 7.5. Where indicated, 13.2 μM Hsp90 was included in the incubation. At time 0 and 15, an aliquot (5.3 μl) was added to an oxyhemoglobin solution to measure nNOS activity. Statistical probability is expressed as *p<0.05, **p<0.01, ***p<0.001.

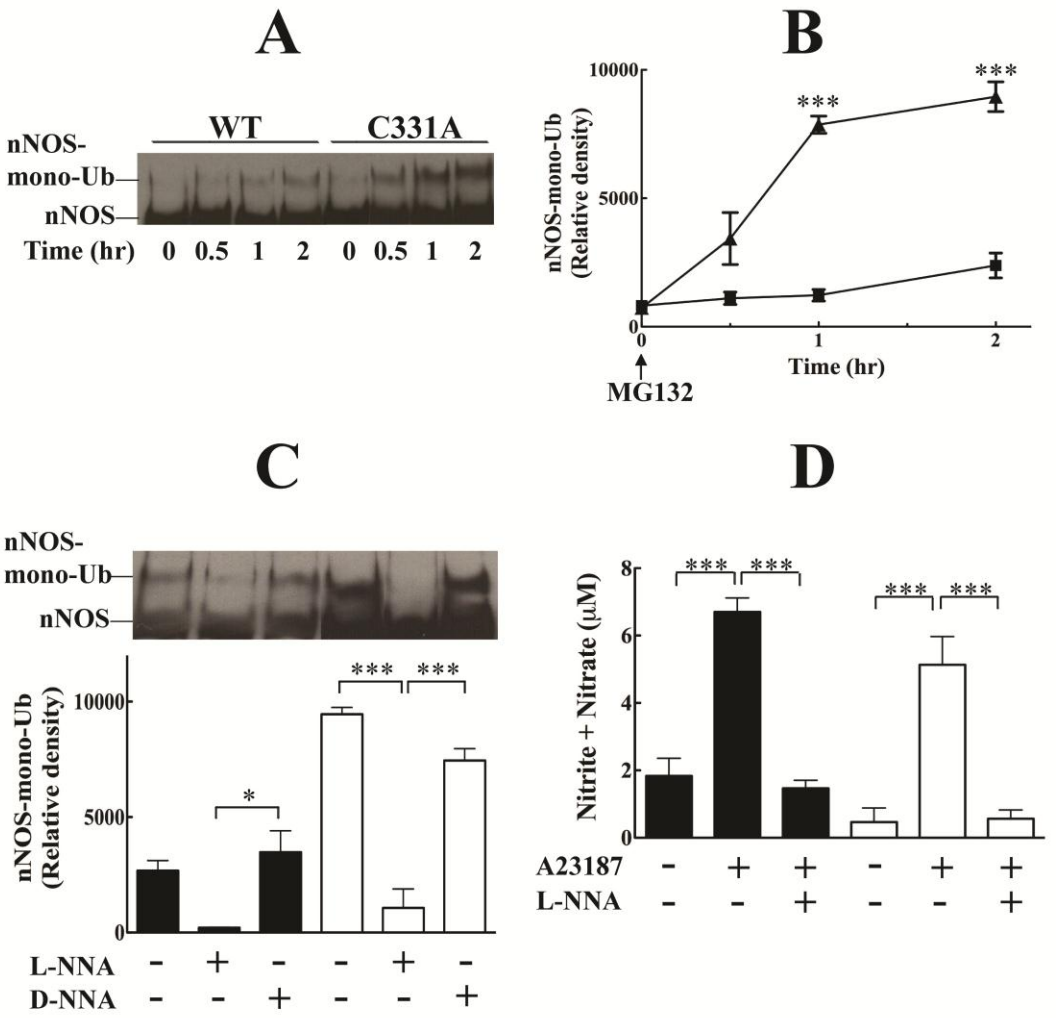

Fig. 3.7. C331A nNOS is preferentially ubiquitinated in HEK293T cells. The ubiquitination of nNOS in HEK293T cells was determined after transient co-transfection with ubiquitin and immunoprecipitation as described in *Experimental Procedures*. *A*, immunoblot showing the ubiquitin conjugate of wild type nNOS (WT) or C331A mutant of nNOS. The time indicates the duration of time the cells were treated with MG132. *B*, the ubiquitin conjugate of wild type (square) or C331A mutant (triangle) of nNOS that was formed in the HEK293T cells was quantified and plotted. *C*, the effect of the treatment of cells with N^G-nitro-L-arginine (L-NNA) or N^G-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 1h with 0.4 mM N^G-nitro-arginine and then treated with MG132 for 2h. *D*, the nitrite and nitrate produced from wild type expressing cells (solid bars) and C331A nNOS expressing cells (open bars). Cells were treated for 1h with 4 μM calcium ionophore (A23187) alone or with 0.4 mM N^G-nitro-L-arginine (L-NNA). The amount of nitrate and nitrite released into the medium was assayed by the Greiss method as described in *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 *p<0.05, ***p<0.001.

ascribed as the monoubiquitin conjugate (10). This major ubiquitin adduct was quantified and plotted in Fig. 3.7B. The C331A nNOS is more readily ubiquitinated in cells (triangles) over that of the wild type nNOS (squares), consistent with observations from in vitro studies. As shown in Fig. 3.7C, the L-isomer of N^G-nitroarginine protects both the wild type (solid bars) and the C331A nNOS (open bars). Again, the D-isomer is not effective at protecting either NOS enzyme, confirming that the active site cleft is important in regulating nNOS ubiquitination. To determine if we have produced functional NOS, we measured the nitrite and nitrate, which are the stable oxidation products of NO, released from the transfected cells after activation with a calcium ionophore. The released nitrite and nitrate is an indirect measure of the activity of the transfected nNOS. The calcium ionophore-mediated production of nitrite and nitrate were similar in the wild type (solid bars) and C331A nNOS (open bars) containing cells (Fig. 3.7D). This is consistent with the comparable specific activities of the purified wild type and C331A nNOS and indicates that we produced functionally active enzyme in the cells. Furthermore, inhibition of the nNOS by N^G-nitro-L-arginine completely blocked the formation of nitrite and nitrate. Although active enzyme is produced, the C331A nNOS must nonetheless become destabilized for ubiquitination.

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 Dr. Masters' laboratory showed that a mutation of cysteine residue 331 of nNOS to an alanine perturbs the L-arginine and tetrahydrobiopterin binding cleft of nNOS (18), 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 play a critical role in dimer stability. The C331A nNOS can exist in

an inactive form, which does not bind tetrahydrobiopterin or L-arginine very well (18). This inactive form of C331A nNOS can be reversibly activated upon incubation in the presence 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 current study. This active form of C331A nNOS had similar Km and Vmax values for L-arginine to that of the wild type nNOS. The expression of C331A nNOS in HEK293T cells catalyzes NO formation after treatment with 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.

The importance of the substrate binding cleft in regulating ubiquitination is also apparent from studies with N-nitroarginine. We show here that N^G-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 N^G-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 triggers 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. Since 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 site in intimate association with the heme and participates in catalysis. In that 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). Crosslinking 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 2D-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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CHAPTER 4

IDENTIFICATION OF THE SITES ON NEURONAL NITRIC OXIDE SYNTHASE TARGETED FOR CHIP-DEPENDENT UBIQUITINATION

Summary

Nitric oxide synthase (NOS), a cytochrome P450-like hemoprotein enzyme, catalyzes the synthesis of nitric oxide, a critical signaling molecule in a variety of physiological processes. Our lab has discovered that certain drugs suicide-inactivate neuronal NOS (nNOS) and lead to the preferential ubiquitination of the inactivated nNOS by an Hsp70- and CHIP (C-terminus of Hsp70-interacting protein)-dependent process. Conversely, calmodulin and certain reversible inhibitors stabilize the nNOS. We wish to understand the process by which altered nNOS is recognized and ubiquitinated. We developed an *in vitro* ubiquitination system that contains purified E1, E2 (UbcH5a), and CHIP that recapitulates the cells' ability to selectively recognize and ubiquitinate the altered forms of nNOS. For example, the slowly reversible inhibitor, N^G-nitro-L-arginine, but not the D-isomer, protects nNOS from ubiquitination. We identified 12 ubiquitination sites through LC-MS/MS analysis of the tryptic peptides obtained from the ubiquitinated nNOS. Nine sites are located in the oxygenase domain, two in the calmodulin-binding domain, and one in the reductase domain. Mutation of the lysines in the calmodulin-binding domain greatly decreases the poly-ubiquitination and degradation

of nNOS, indicating that this region plays an important role in regulating nNOS turnover. Understanding the exact site of ubiquitination is an important step in determining the process by which a protein becomes recognized and degraded by the ubiquitin-proteasome system.

Introduction

Nitric oxide synthase (NOS) is a cytochrome P450-like hemoprotein enzyme that catalyzes the conversion of L-arginine to L-citrulline and nitric oxide, which is an important cellular signaling molecule (1). There are three major NOS isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NOS is active as a homodimer, and activity requires the presence of bound heme, tetrahydrobiopterin, and L-arginine in the oxygenase domain, as well as FMN, FAD, and NADPH, which bind in the reductase domain (2). Ca^{2+} and calmodulin are also required for activity, and this is one of the many methods by which this enzyme is regulated. Another type of regulation of NOS occurs through the ubiquitination and proteasomal degradation of the enzyme (3).

Ubiquitination is carried out by three ubiquitinating enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase. CHIP (C-terminus of Hsc70-interacting protein) is a specific E3 ligase that has been characterized as a major protein involved in the ubiquitination of all NOS isoforms (4-6), as well as cytochromes P450 3A4 and 2E1 (7, 8). There is some redundancy in E3 ligases that ubiquitinate nNOS, as indicated by the equivalent ability of CHIP-/- and CHIP +/+ mouse embryonic

fibroblasts to ubiquitinate nNOS (9), however, CHIP has been shown to be the primary E3 ligase for nNOS in the *in vitro* ubiquitination system of reticulocyte lysate (10, 11).

Certain drugs have been shown to cause the suicide inactivation, covalent alteration in the heme/substrate-binding cleft, ubiquitination, and proteasomal degradation of nNOS (12-15). Thus, the heme/substrate-binding cleft appears to control the ubiquitination of nNOS (3, 16). A C331A mutant of nNOS, which has an altered active site and is unstable, is preferentially ubiquitinated compared to the wildtype enzyme, and serves as a model for a suicide-inactivated nNOS (10). N^G-nitro-L-arginine, a slowly reversible inhibitor, protects both the wildtype and C331A mutant from ubiquitination (10). This process is specific for the heme active site, since the D-isomer had no effect on ubiquitination, thus showing that certain ligands can stabilize a conformation of C331A nNOS that is resistant to ubiquitination (10). It is clear that the ligand-binding cleft plays an important role in nNOS triage; however, it is unknown what regions of the unstable C331A nNOS are recognized to be targeted for ubiquitination and degradation.

CHIP binds through an amino-terminal tetratricopeptide repeat (TPR) domain to both Hsp70 and Hsp90 (17, 18). The C331A mutant nNOS, as well as suicide-inactivated nNOS are both ubiquitinated by an Hsp70/CHIP-mediated process, and Hsp90 opposes this ubiquitination (4, 10, 15). Moreover, the addition of Ca^{2+}/calmodulin and Hsp90 to an *in vitro* ubiquitination system protects nNOS from ubiquitination (15). In HEK293 cells stably expressing individual nNOS domains (oxygenase domain, oxygenase-calmodulin binding domain, and reductase domain), Hsp90 and Hsp70 were both found in heterocomplexes with fragments containing the oxygenase domain, which contains the

ligand-binding cleft (19). Moreover, the stabilizing ligand N^G-nitro-L-arginine decreased the chaperone association with the nNOS fragments (19).

The role of chaperones in nNOS ubiquitination has been extensively studied, yet it is still unknown what region on nNOS is recognized for ubiquitination. Wang *et al.* recently found that ubiquitination of CYP 2E1 occurs in regions containing phosphorylated Ser/Thr residues, as well as surface clusters of Asp/Glu (20). Destabilization of the nNOS dimer has been shown to enhance the ubiquitination of nNOS (13), and it has been suggested that destabilization of the dimer could lead to exposure of hydrophobic residues normally hidden in the active form of the protein (13). Characterization of the site of ubiquitination of nNOS may aid in deciphering the trigger that selectively culls dysfunctional nNOS for ubiquitination.

Previous *in vitro* studies successfully show the ubiquitination of nNOS (4, 10, 15). Here we maximize the conversion of nNOS to the ubiquitinated form, by altering the ratio of nNOS to the ubiquitination proteins, in order to purify ubiquitinated nNOS for further analysis. Through tryptic digestion and analysis by LC-MS/MS, we found 12 sites of nNOS ubiquitination. Nine of these sites are located in the oxygenase domain of nNOS, two are located in the calmodulin-binding region, and one is located in the reductase domain. Furthermore, we confirmed the role of the calmodulin-binding region in nNOS ubiquitination by creating a mutant nNOS (7R) that has all lysines in the calmodulin-binding region mutated to arginines. The polyubiquitination of this nNOS 7R mutant was significantly decreased compared to the wildtype enzyme. Moreover, degradation of the nNOS 7R mutant was attenuated compared to the wildtype enzyme.

Thus, ubiquitination in the calmodulin-binding region is one of the signals for selective degradation by the proteasome.

Experimental Procedures

Materials. (6R)-5,6,7,8-Tetrahydro-L-biopterin (BH4) was purchased from Dr. Schirck's Laboratory (Jona, Switzerland). Protein A-Sepharose, ubiquitin, ATP, creatine phosphokinase, L-arginine, N^G-nitro-L-arginine, N^G-nitro-D-arginine, and rabbit polyclonal anti-nNOS were purchased from Sigma (St. Louis, MO). Goat anti-rabbit IRDye was purchased from Licor Biosciences (Lincoln, NE). His-tagged ubiquitin activating enzyme (E1), K0R ubiquitin, K6R ubiquitin, K11R ubiquitin, K27R ubiquitin, K29R ubiquitin, K33R ubiquitin, K48R ubiquitin, and K63R ubiquitin were from R&D Systems (Minneapolis, MN). Creatine phosphate was from Fluka (St. Louis, MO). Complete Mini protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN). Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was from QIAGEN Inc (Valencia, CA). The cDNA for rat neuronal NOS was kindly provided by Dr. Solomon Snyder (Johns Hopkins Medical School, Baltimore, MD). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (Univ. of Michigan). The cDNA for expressing the UbcH5a−GST fusion protein was kindly provided by C. M. Pickart (Johns Hopkins Medical School, Baltimore, MD). pET30a CHIP plasmid for expressing His-CHIP was kindly provided by C. Patterson (University of North Carolina, Chapel Hill, NC). Nickel magnetic beads and Amicon Ultra centrifugal spin filters were from Millipore (Billerica, MA). Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). ^{125}I-

Labeled antibody against rabbit IgG was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). DE52 was purchased from Whatman (Clifton, NJ).

Expression and Purification of nNOS, C331A nNOS, 7R nNOS, Hsp70, Hsp40, GST-tagged UbcH5a, His-tagged ubiquitin, and CHIP . The C331A construct in pCW was made using the site-specific mutagenesis approach described by Martasek *et al*. (21). The C331A-nNOS and wild type nNOS pCW plasmid was transfected as described (22), except that BL21 E. coli cells were used. The bacterially expressed wild type and C331A nNOS were purified by 2'5'-ADP Sepharose and mono Q ion exchange chromatography as described (23), except that 10 µM BH4 was present in the buffers used during purification. The enzyme as isolated was active (V_{max} of 443 nmol/min/mg protein) and further incubation with L-arginine did not increase the activity. To make the 7R mutant nNOS, we mutated all 7 lysine residues present in the calmodulin binding region of nNOS (residues 725, 732, 733, 739, 743, 751, and 754) to arginine residues using QuikChange Multi Site-Directed Mutagenesis Kits (Stratagene, La Jolla, CA). The three 5'- phosphorylated primers used were 5'-CCCCCACGAGGCGGCGAGCTATCGGCTTTAGGAGATTGGCAGA-3', 5'-GGCCGTCAGGTTCTCAGCCAGGCTAATGGGACAG-3', and 5'-GCCATGGCCAGGAGGGTCAGGGCGACCATTCTCTAC-3' (codons for arginine are underlined). The template was pCWnNOS. The plasmid with the desired mutation was confirmed by sequencing and digested with *Pfl*M1 to liberate the 1863 bp fragment, then subcloned into *Pfl*M1 sites of the wt-pCWnNOS vector. The resultant construct was designated as pCW7R. The nNOS mutants (including a wild type as control) were bacterially expressed using the pCW vector and BL21 (DE3) competent cells

(Stratagene), according to the manufacturer's recommendations. Cells from 1L-cultures were harvested 48 h after induction with IPTG, and ruptured by french press at 1500 PSI. The lysates were processed and purified as above for the Sf9 cells, except that a PD-10 gel filtration column (GE Healthcare) was used instead of the Sephacryl S-300 HR gel filtration column. The samples were concentrated with the use of a Centriplus YM-100 concentrator and stored at –80 ^{o}C. The 7R nNOS mutant was found to be active (Vmax 7R nNOS: 195 nmol/min/mg protein).

His-CHIP, his-tagged human Hsp70, and his-ubiquitin were bacterially expressed and purified by Ni-NTA affinity chromatography as previously described (17). YDJ-1, the yeast ortholog of Hsp40, was expressed in bacteria and purified by sequential chromatography on DE52 and hydroxylapatite as described previously (24). GST-tagged UbcH5a (E2, ubiquitin carrier protein) was bacterially expressed and purified by GSH-Sepharose affinity chromatography as described (25).

In Vitro Ubiquitination of nNOS by Purified Ubiquitinating System. The nNOS was pretreated with Hsp70 and Hsp40 and then ubiquitinated. In these experiments, 60 µM Hsp70 and 3 µM Hsp40 were incubated for 30 min at 30 ^{o}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 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, 5mM 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 at 30 ^{o}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 containing 5% (w/v) SDS, 20% (v/v) glycerol, 6 mg/ml DTT, and 0.02% (w/v) bromophenol blue in 125 mM Tris-HCl, pH 6.8 was added and an aliquot (14 µl) was loaded for Western blotting.

SDS-Polyacrylamide Gel Electrophoresis 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 volts. The blot was probed with anti-nNOS (0.01%, w/v) and then an infrared goat anti-rabbit secondary antibody (0.0067%, w/v) and the immunoreactive bands were visualized with the use of the Licor Odyssey Imaging System, and the bands were quantified using Image Studio software (Licor). The linearity of the system was verified measuring the signal of various concentrations of nNOS.

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 five-fold to 20 µM. The large scale preparation consisted of a total volume of 800ul of the ubiquitination reaction mixture. After the 1 h incubation at 30 ^{o}C, the sample was concentrated to 40 µl in 100K spin filters to remove unconjugated ubiquitin and other smaller proteins. This sample was then resuspended with 1 mL of Equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, pH 8) 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 ^{o}C. The supernatant was then removed, and the sample was washed three times with 1 mL Wash buffer (50 mM sodium phosphate, 300

mM sodium chloride, 10 mM imidazole, pH 8). 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 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 MSBioworks (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 dithiothreitol at 60 ^{o}C followed by alkylation with 50 mM iodoacetamide at room temperature. Samples were then digested with trypsin (Promega) at 37 ^{o}C for 4 hours. 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. 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 fifteen most abundant ions were selected for MS/MS. The MS/MS data were searched against the SwissProt database using Mascot search engine (Matrix Science, London, UK; version Mascot). Mascot was set up to search the ipi.RAT.v3.75.decoy database assuming the digestion enzyme trypsin. S-carbamoylmethylcysteine cyclization (N-terminus) of the n-terminus, deamidation of asparagine and glutamine, oxidation of methionine, acetylation of the n-terminus, and ubiquitination residue of lysine were specified in Mascot as variable modifications.

Peptides that were observed in at least two independent scans, with a probability greater than 95% were selected as putative nNOS ubiquitination sites. Of these, the sites were confirmed by manual inspection of the raw MS/MS spectra.

In vitro degradation of nNOS by Fraction II - Fraction II was prepared from rabbit reticulocyte lysate as previously described (11). 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 dithiothreitol, 50 μM ubiquitin, an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, 5 mM $MgCl_2$, and 10 units/ml creatine phosphokinase), 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 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 x 8 cm). Proteins were then transferred to nitrocellulose membranes (0.2 μm, BioRad) and probed with 0.1% anti-nNOS IgG. The immunoblots were then incubated a second time with ^{125}I-conjugated goat anti-rabbit IgGs to visualize the immunoreactive bands. The membranes were dried and exposed to a phosphor imaging screen for 4 hours, digitized using a Typhoon™ imaging system. Individual nNOS bands were selected, baseline corrected, and quantified using ImageQuant™ software.

Results

Ubiquitination of nNOS by a Purified System Containing CHIP. As shown in Fig. 1A, incubation of Hsp70-pretreated nNOS with a purified ubiquitination system

containing E1, E2, and CHIP gives the time-dependent formation of higher molecular mass nNOS-bands, which we have previously described as nNOS-ubiquitin conjugates (14). Similar bands have been visualized by anti-ubiquitin after immunoprecipitation with anti-nNOS, or with the use of ^{125}I-ubiquitin (14). Based on the estimated molecular mass calculated from the migration of the bands against the 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 incubation time, are poly-ubiquitinated products (*poly-Ub*). As shown in Fig. 1B, the loss of nNOS and the formation of either mono-Ub (*gray bars*) or poly-Ub (open bars) are dependent on ubiquitin (-Ub). The deletion of CHIP also completely prevents the formation of nNOS-ubiquitin conjugates (-CHIP). As shown in Fig. 1C, 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 timepoint (*open squares*) remain 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 (4, 10, 15), the current system gives a much higher yield (~50%) of ubiquitinated nNOS.

Validation of Regulated Ubiquitination of nNOS by Purified CHIP. Although it was important to achieve a high yield of nNOS-ubiquitin conjugates for the mapping studies described below, it was equally important to assess whether the current ubiquitin conjugation system maintained the biological fidelity found in cells. The nNOS is protected from ubiquitination by the L- but not the D-isomer of N^G-nitro-arginine (NNA),

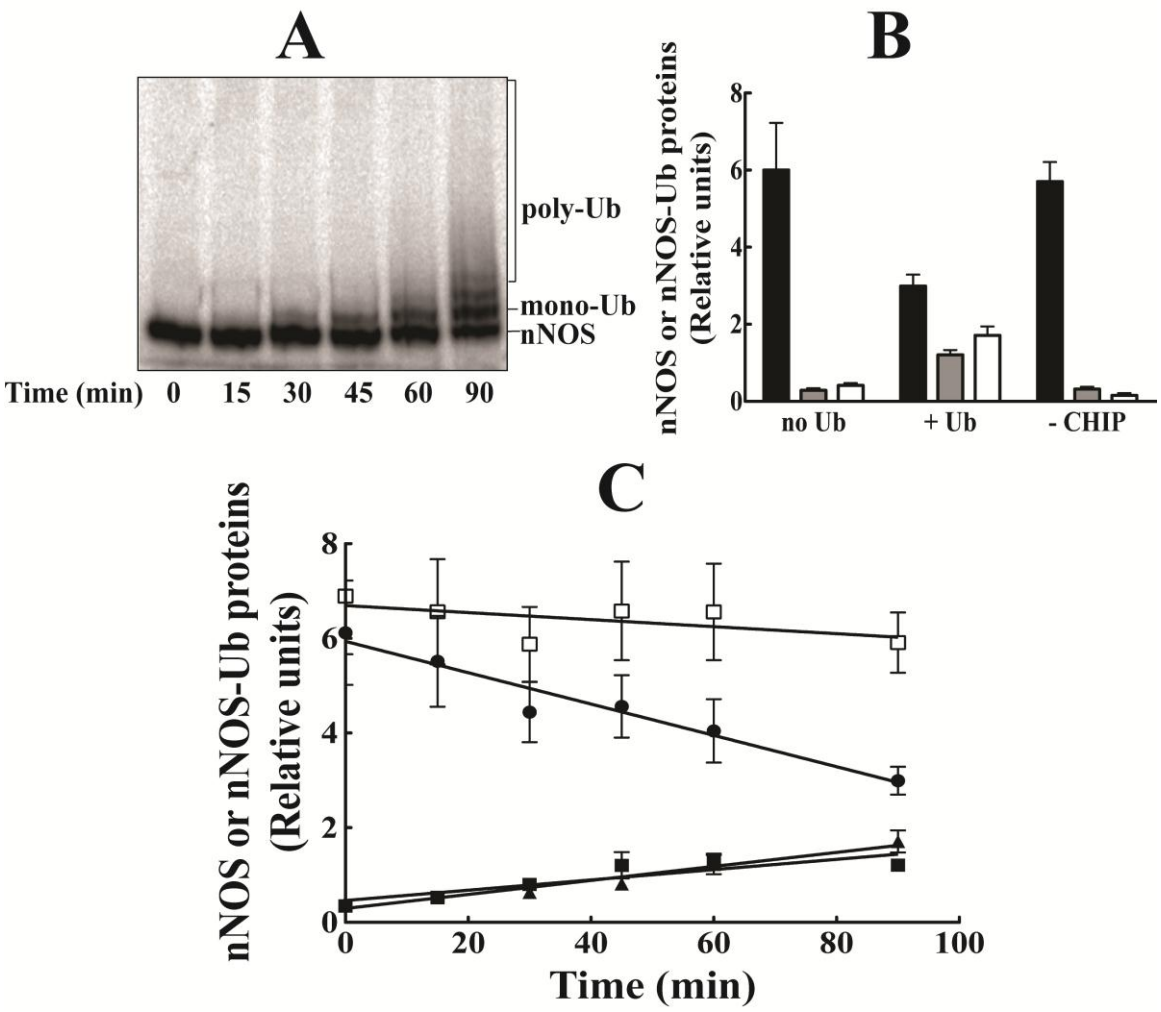

Figure 4.1. Ubiquitination of nNOS in a purified system is CHIP- and time-dependent. The ubiquitination of nNOS by a purified ubiquitination system and Western blots for ubiquitinated nNOS is described under "Experimental Procedures." *A,* Time-dependent formation of nNOS-ubiquitin conjugates. nNOS, mono-ubiquitinated nNOS (mono-Ub), and poly-ubiquitinated nNOS (poly-Ub) are indicated. *B,* Ubiquitin and CHIP are required for nNOS ubiquitination. The addition of his-ubiquitin (Ub) or the subtraction of CHIP on nNOS (black bars), mono-Ub (gray bars), and poly-Ub (white bars) levels after a 60 min incubation in the purified ubiquitination system. The values are the mean ± S.E. (n = 4). *C,* quantification of nNOS and ubiquitinated nNOS from the blot shown in *A.* nNOS (closed circles), mono-ubiquitinated nNOS (closed squares) and poly-ubiquitinated nNOS (closed triangles) were quantified. The sum of all bands is also shown (open squares). The values are the mean ± S.E. (n = 4).

in intact cells and in *in vitro* studies with cell lysates (10, 13). Thus, the ubiquitination machinery can discern conformational changes about the active site (16). As shown in Fig. 2A, the pretreatment of nNOS with L-NNA, but not D-NNA, diminishes the nNOS ubiquitin conjugates. The quantification of the nNOS bands clearly shows that L-NNA protects the native nNOS from ubiquitination (Fig. 2B, *nNOS, cf. lane 2* with *3*), whereas the D-NNA has no significant effect (*cf. lane 2* with *4*). Consistent with the effects on nNOS, there is a significant decrease in the amount of both mono (*mono-Ub*)- and poly (*poly-Ub*)- ubiquitinated nNOS when L-NNA, but not D-NNA, 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.

To further validate the identity of the nNOS mono-ubiquitin and poly-ubiquitin bands identified above, we used a form of ubiquitin that has all seven lysines mutated to arginines (K0R). This mutant ubiquitin is unable to form ubiquitin-ubiquitin polymers (*poly-Ub*). As shown in Fig. 4.3A, nNOS poly-ubiquitin conjugates are not apparent with the K0R ubiquitin (*cf. lanes 5-7* with *lanes 1-4*). The K0R ubiquitin does support the formation of two nNOS-ubiquitin bands above the nNOS that we have labeled as mono-ubiquitinated nNOS (*mono-Ub)* and di-ubiquitinated nNOS (*di-Ub*). The indicated bands were quantified and plotted in Fig. 4.3B. As seen by the disappearance of the starting nNOS band, the ubiquitination of nNOS is much slower in the presence of the K0R ubiquitin (*nNOS, cf. gray bars* with *open bars*). The mono-ubiquitin form accumulates to the same extent with the wildtype and K0R ubiquitin (*mono-Ub, cf. gray bars* with *open bars*). However, there is no poly-ubiquitin formed with the K0R ubiquitin (*poly-Ub, cf.*

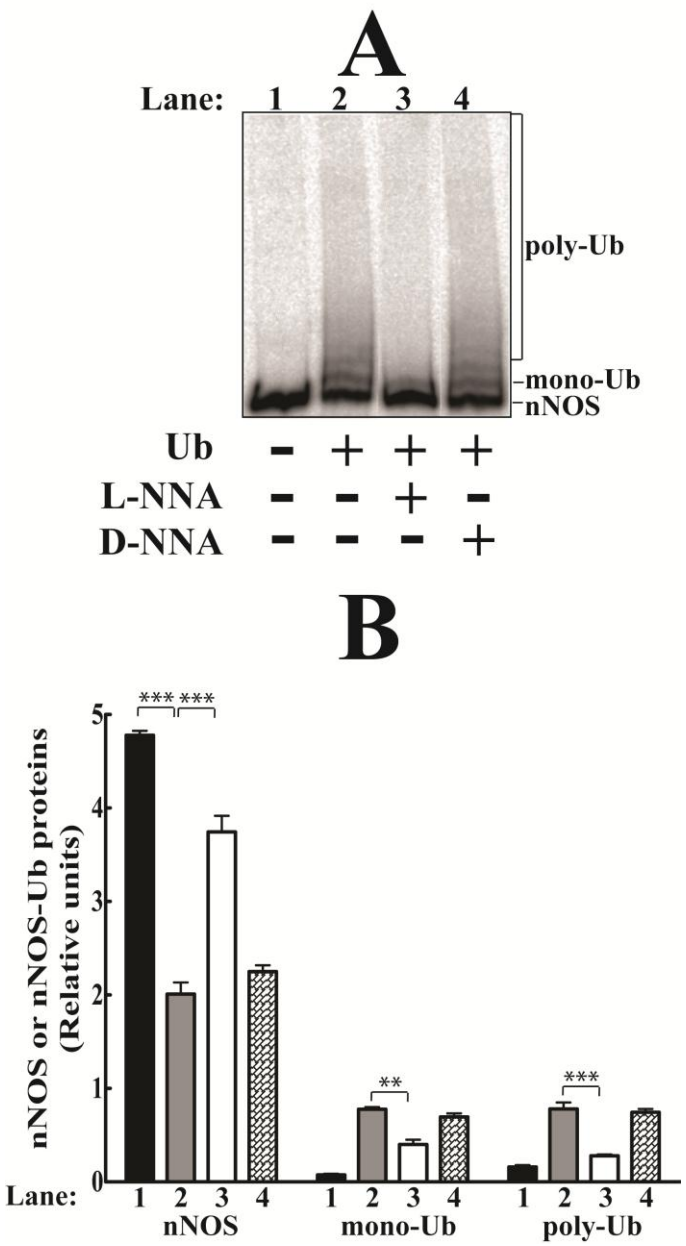

Figure 4.2. N^G-nitro-L-arginine stereospecifically decreases both mono- and poly-ubiquitination of nNOS. Ubiquitination of nNOS was performed as described under "Experimental Procedures." nNOS was treated with N^G-nitro-L-arginine (L-NNA) or the stereoisomer N^G-nitro-D-arginine (D-NNA) for 60 min, and the effects on ubiquitination were measured. *A,* a representative blot is shown. *B,* quantification of ubiquitination corresponding to the blot shown in *A.* Closed bars, ubiquitin was omitted; gray bar, ubiquitin included; open bars, L-NNA was added; hashed bars, D-NNA was added. The values are the mean ± S.E. (n = 4). Statistical probability is expressed as follows: **, $p<0.01$; ***, $p<0.001$.

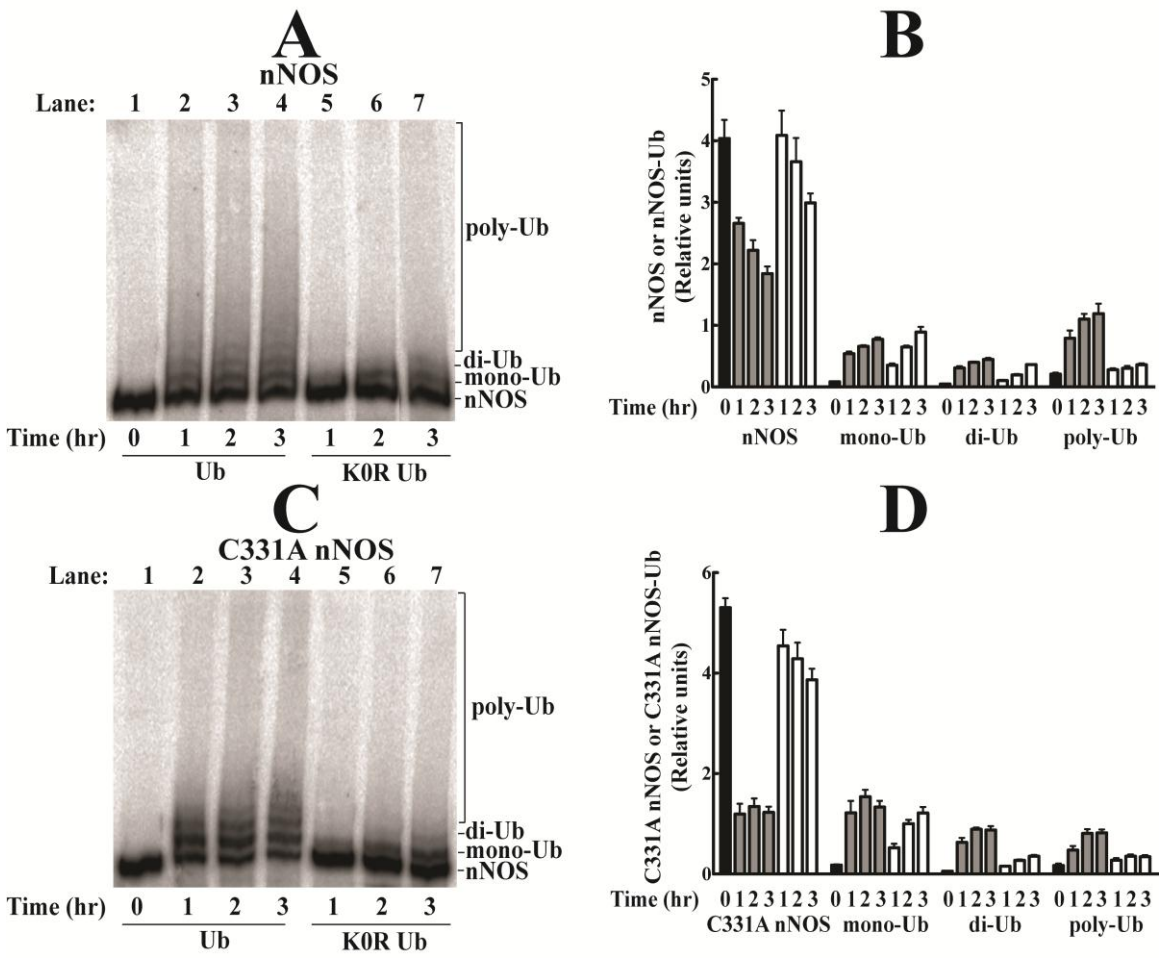

Figure 4.3. Effect of Ubiquitin or K0R Ubiquitin on Ubiquitination of nNOS or C331A nNOS. The ubiquitination of nNOS and C331A mutant nNOS by a purified ubiquitination system was performed as described under "Experimental Procedures." nNOS, mono-ubiquitinated nNOS (mono-Ub), di-ubiquitinated nNOS (di-Ub) and poly-ubiquitinated nNOS (poly-Ub) are indicated. *A*, nNOS ubiquitination was produced at the indicated timepoints in the presence of WT ubiquitin (WT Ub) or lysineless mutant ubiquitin (K0R), which cannot form poly-ubiquitin chains. *B*, quantification of nNOS ubiquitination corresponding to the blot shown in *A.* Closed bars, 0 min timepoint; gray bars, WT ubiquitin; open bars, K0R ubiquitin. The values are the mean ± S.E. (n = 4). *C*, C331A nNOS ubiquitination was produced at various timepoints in the presence of WT ubiquitin (WT Ub) or lysineless mutant ubiquitin (K0R). *D*, quantification of C331A nNOS corresponding to the blot shown in *C.* Closed bars, 0 min timepoint; gray bars, WT ubiquitin; open bars, K0R ubiquitin. The values are the mean ± S.E. (n = 4).

gray bars with *open bars*). Interestingly, the di-ubiquitin band does increase in the K0R samples, suggesting that it likely contains nNOS that was successively mono-ubiquitinated twice. We wondered if the lack of formation of poly-ubiquitinated nNOS was due to a very slow ubiquitination reaction in the presence of K0R ubiquitin, even though we extended the reaction time in this study. To further attempt to address this, we substituted nNOS for a C331A mutant of nNOS, which we have previously shown to be more susceptible to ubiquitination in cells and in *in vitro* systems (10). As shown in Fig. 3C, the C331A appeared to be ubiquitinated in a similar manner to that of nNOS with wildtype (*Ub*) and K0R ubiquitin. The C331A nNOS is ubiquitinated at a faster and greater extent than nNOS, with nearly 75% converted after the first hour of incubation (Fig. 3D, *C331A nNOS, gray bars*). However, ubiquitination of C331A nNOS was also very slow in the presence of K0R ubiquitin (*C331A nNOS, open bars*). Although the mono-ubiquitin band (*mono-Ub*) was formed under all conditions, again no poly-ubiquitin bands could be found with K0R (*poly-Ub, cf. gray bars* with *open bars*). The di-ubiquitin band of C331A nNOS in the K0R-ubiquitin sample increased over time but never reached the level seen in samples containing the wildtype ubiquitin (*di-Ub, cf. gray bars* with *open bars*), suggesting that it likely is a mixture of mono- and poly-ubiquitinated forms. For simplicity, the di-Ub band was not included in further quantification.

Nature of the Ubiquitin Linkages in nNOS Poly-Ubiquitin Conjugates. As seen above, when all seven lysine residues on ubiquitin are mutated to arginine (K0R mutant), the poly-ubiquitin chains do not form. In order to further examine the types of ubiquitin linkages that contribute to poly-ubiquitination of nNOS, we used various single residue

mutants of the seven lysine residues of ubiquitin (K6R, K11R, K27R, K29R, K33R, K48R, K63R). As shown in Fig. 4.4A, these single residue mutants were compared to the K0R mutant and wildtype ubiquitin (*WT*). As expected, all of the mutant ubiquitins could support the formation of the mono-ubiquitinated nNOS (*mono-Ub*), but not the poly-ubiquitinated nNOS (*poly-Ub*). As shown in Fig. 4.4B, the poly-ubiquitinated nNOS was quantified and plotted. The K6R, K33R, and K63R mutant ubiquitins decreased the poly-ubiquitination of nNOS to the level seen for K0R. The K27R, K29R, and K48R ubiquitins also greatly decreased the level of poly-ubiquitination, whereas the K11R ubiquitin only decreased the poly-ubiquitination by 30%. Thus, it appears that the poly-ubiquitination of nNOS can occur with any lysine residue, but predominantly all lysines except K11.

Identification of nNOS Ubiquitination Site(s) by LC-MS/MS Analyses. The ubiquitination system described above was scaled up by a factor of 40 to generate enough of the ubiquitinated nNOS conjugates for analysis by LC-MS/MS. The reaction mixture was initially filtered through a 100 kDa molecular sieve, purified by magnetic Ni-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 signature ubiquitin remnant (GG) attached to the nNOS peptide (26). nNOS was identified with 81% sequence coverage. Several ubiquitination sites on nNOS were identified by this approach, as shown in Table 1. Nine of these sites are located in the oxygenase domain of nNOS (residues 1-724), two are located in the calmodulin-binding region (725-757), and one is located in the reductase domain (residues 758-1433).

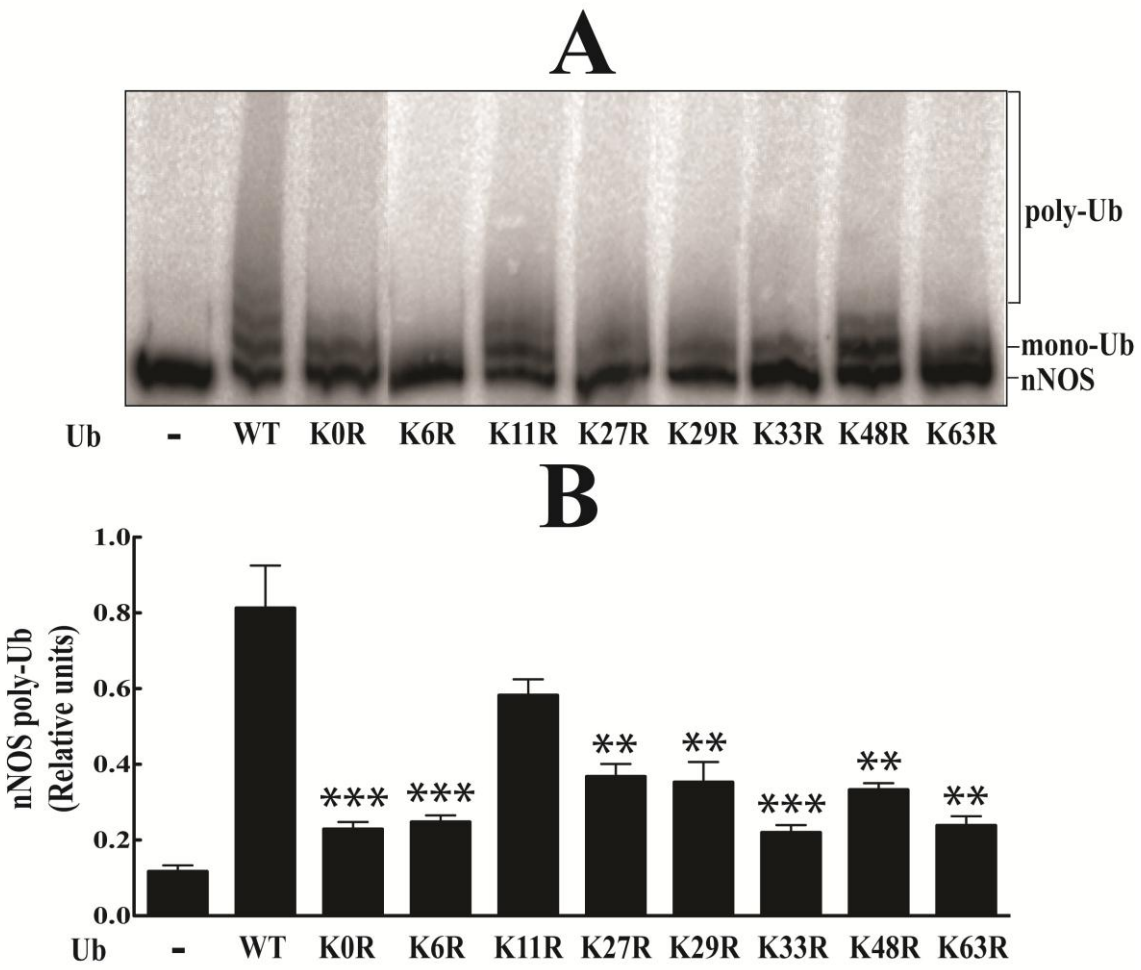

Figure 4.4. Poly-ubiquitination of nNOS occurs through several different lysine-linked chains on ubiquitin. Ubiquitination of nNOS was performed for 5 hours using various mutant ubiquitins, each with a different lysine mutated to an arginine (K6R, K11R, K27R, K29R, K33R, K48R, K63R), as well as a lysine-less ubiquitin (K0R) and wild-type ubiquitin (WT). A representative blot is shown in *A. B,* quantification of poly-ubiquitination under the conditions in *A*. The values are the mean ± S.E. (n = 3). Statistical probability is expressed as follows: **, $p<0.01$; ***, $p<0.001$.

Site	Sequence	m/z	z	Mascot Ion Score
K24	K(GG)VGGLGFLVK	377.9	3	57.59
K33	VGGLGFLVK(GG)ER	430.3	3	62.82
K143	AVDLSHQPSASK(GG)DQSLAVDR	746.7	3	58.93
K188	STK(GG)ANLQDIGEHDELLK	675.7	3	27.93
K225	GGPAK(GG)AEM(ox)KDTGIQVDR	635.0	3	43.08
K229	AEMK(GG)DTGIQVDR	492.9	3	54.26
K245	DTGIQVDRDLDGKSHK(GG)	633.3	3	51.93
K370	EFLDQYYSSIK(GG)R	554.9	3	31.48
K386	LEEVNK(GG)EIESTSTYQLK	709.0	3	36.78
K739	LAEAVK(GG)FSAK	589.3	2	67.37
K743	FSAK(GG)LMGQAMAK	698.9	2	52.83
K1371	IMTQQGK(GG)LSEEDAGVFISR	741.7	3	63.93

Table 4.1. nNOS ubiquitination sites. Large-scale ubiquitination of nNOS and subsequent purification of the mono-ubiquitinated nNOS is as described in "Experimental Procedures." Tryptic peptides obtained from the sample were analyzed by nano LC/MS/MS. The fifteen most abundant ions from each scan were selected for MS/MS. The sequences were then searched against the SwissProt database using the Mascot search engine. Peptides that were observed in at least two independent scans, with a probability greater than 95% were selected as putative nNOS ubiquitination sites. Of these, the sites were confirmed by manual inspection of the raw MS/MS spectra. The abbreviations used are as follows: ox, oxidation.

Of the identified ubiquitination sites, lysine 739 had the highest mascot score (67.37). This residue and lysine 743 (mascot score 52.83) are located in the calmodulin-binding domain of nNOS. Previous studies have indicated the ability of calmodulin binding to block the ubiquitination of nNOS (15). Due to this fact, the MS/MS spectra were more closely analyzed for the residues that are located in the calmodulin-binding region. As shown in Fig. 5A, the spectrum for lysine 739 identifies an addition of 114 Da to the peptide between the high intensity ions y_4 and y_5, which correspond to the addition of the signature ubiquitin remnant (GG) to the peptide. The 114 Da addition can also be seen in the spectrum for lysine 743 (Fig. 5B) between the ions y_8 and y_9.

Role of Calmodulin-Binding Region in Ubiquitination of nNOS. Since we found two sites of ubiquitination located in the calmodulin-binding region of nNOS, we decided to construct a mutant nNOS (7R) that replaced the seven lysines found within the calmodulin-binding region with arginine residues. All seven lysine residues were mutated to investigate the role of this region in ubiquitination. As shown in Fig. 6A, the poly-ubiquitination (*poly-Ub*) of nNOS 7R mutant is significantly decreased compared to the wildtype nNOS (*poly-Ub, cf. black bars* with *open bars*). We then tested the degradation of the wildtype nNOS and nNOS 7R mutant in a more physiologically relevant system containing Fraction II, which is the DE52-retained fraction of reticulocyte lysate that contains all ubiquitinating enzymes and the proteasome. As shown in Fig. 6B, the nNOS 7R mutant (*7R*) degradation is greatly attenuated compared to the wildtype (*WT*) enzyme which can be seen in the Western blot measuring the loss of nNOS protein (*cf. 7R* with *WT*) and the quantification below (*cf. squares* with *circles*).

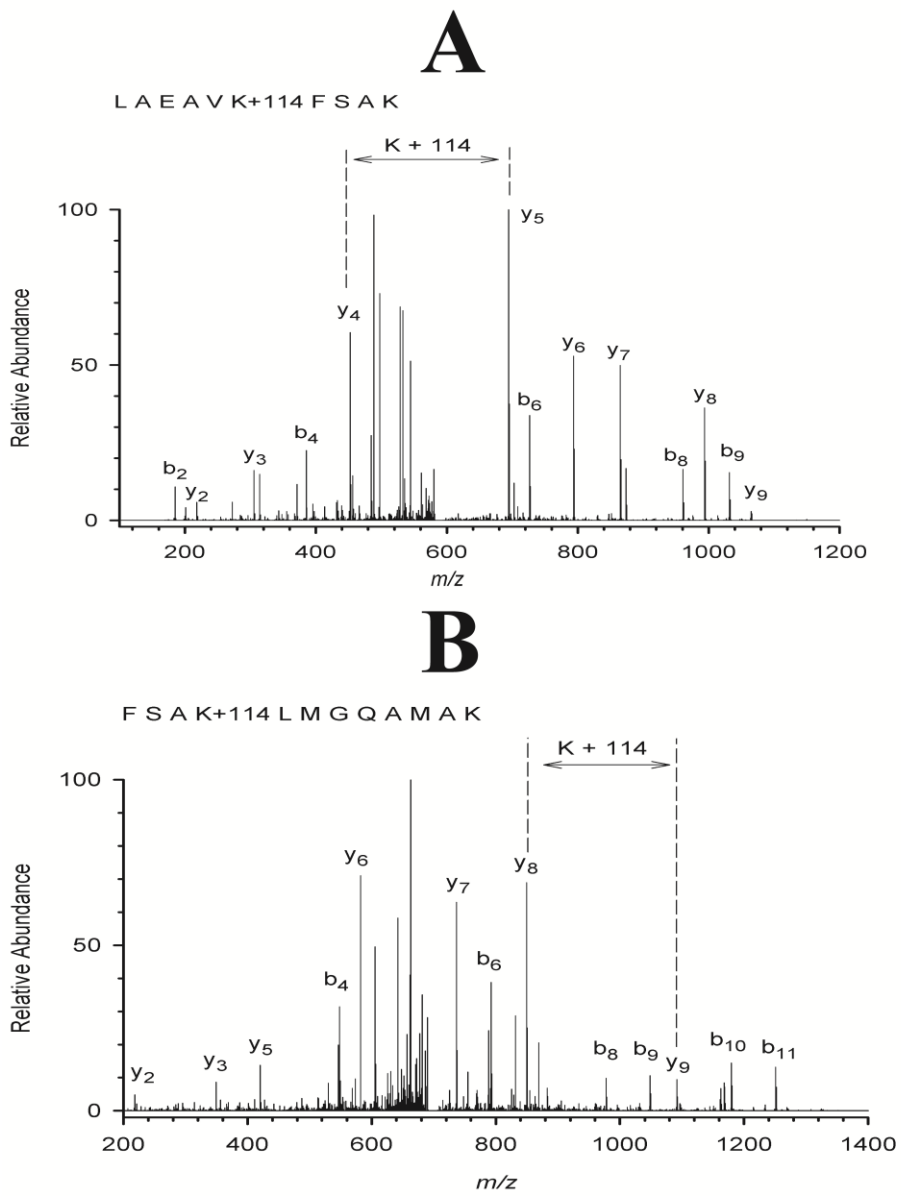

Figure 4.5. MS/MS spectra for validated ubiquitinated residues, K739 and K743. Samples were analyzed as described in "Experimental Procedures." The ubiquitinated peptides were confirmed by manual inspection of the raw MS/MS spectra. The spectra were analyzed for the diagnostic loss of GG as annotated. The y and b ions are labeled that precisely define the site of ubiquitination. *A*, MS/MS spectrum for residue K739, with a Mascot score of 67.4. *B*, MS/MS spectrum for residue K743, with a Mascot score of 52.8.

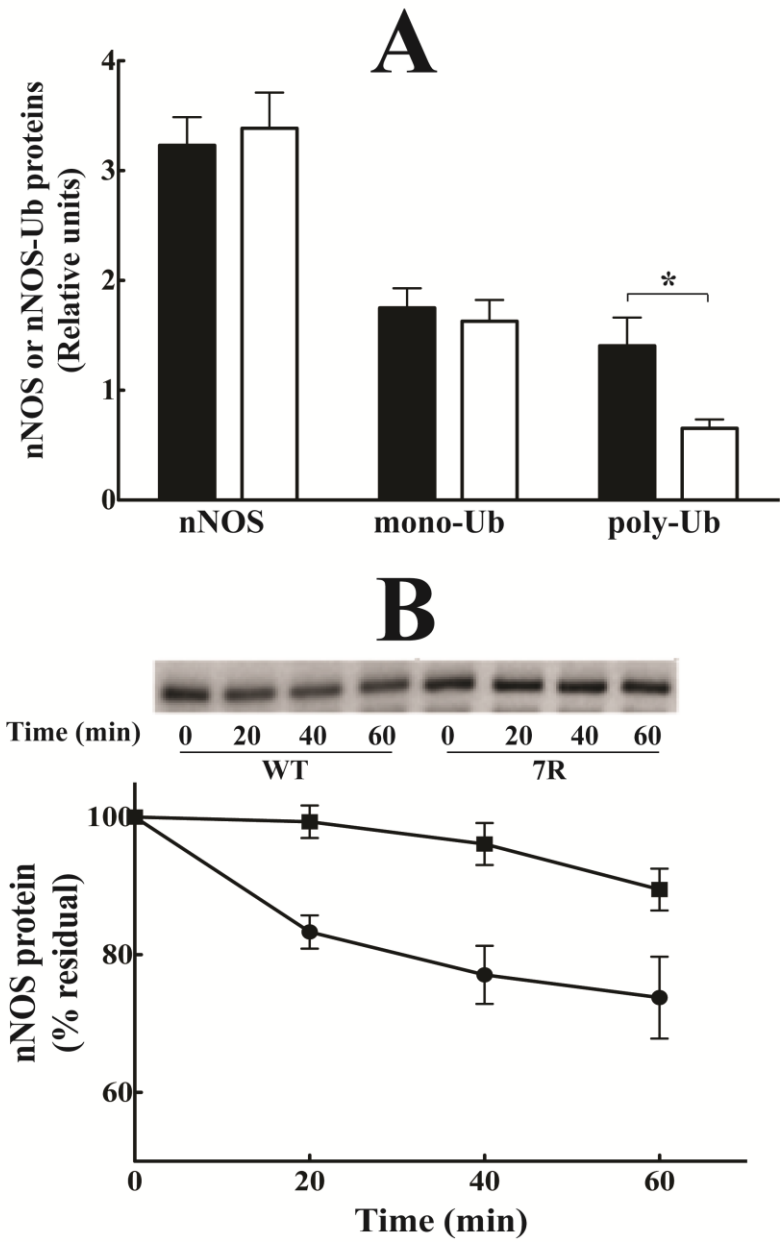

Figure 4.6. **Decreased polyubiquitination (A) and proteasomal degradation (B) of a mutant of nNOS (nNOS 7R) that lacks lysine residues in the calmodulin binding site.** *A*, Ubiquitination of nNOS and nNOS 7R was carried out as described in "Experimental Methods." Samples were ubiquitinated for one hour and analyzed by Western blotting techniques. nNOS, closed bars; nNOS 7R, open bars. The values are the mean ± S.E. (n = 3). *B*, Degradation of nNOS and nNOS 7R in fraction II was examined as described in "Experimental Procedures." Samples were collected at the indicated timepoints and analyzed by Western blot. Quantification is shown below the blots. nNOS, circles; nNOS 7R, squares. The values are the mean ± S.E. (n = 3).

These data indicate that the decreased poly-ubiquitination of the nNOS 7R mutant translates to decreased degradation. Thus, ubiquitination in the calmodulin-binding region must be a signal for selective degradation by the proteasome.

Discussion

The heme- and ligand-binding cleft in the oxygenase domain is an important regulator of nNOS ubiquitination (16). We have previously shown that suicide inactivation of nNOS leads to covalent alteration of the cleft and promotes ubiquitination of nNOS (12, 15). A mutation of cysteine residue 331 of nNOS to an alanine perturbs the L-arginine and tetrahydrobiopterin-binding cleft of nNOS, which destabilizes the enzyme and causes it to be more susceptible to ubiquitination in *in vitro* and cellular studies (10, 21). This C331A mutant of nNOS, as well as the wildtype nNOS, are stabilized by the slowly reversible inhibitor, N^G-nitro-L-arginine (10). Furthermore, we found that Hsp70 and Hsp90 bind the oxygenase domain of nNOS, and this chaperone association decreases in the presence of N^G-nitro-L-arginine (unpublished data). While it is clear that the active site cleft is playing a role in regulation and stability of nNOS, it is unknown where ubiquitination occurs on nNOS, and therefore what structural features of nNOS are available for recognition and subsequent targeting of nNOS. We developed an *in vitro* ubiquitination assay containing purified E1, E2, and CHIP, that recapitulates the cleft-regulated formation of ubiquitinated nNOS. We utilized this system to generate large quantities of ubiquitinated nNOS for LC-MS/MS analysis of tryptic nNOS peptides. We established that ubiquitination of nNOS occurs primarily in 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. The identification of the ubiquitination sites in the oxygenase domain confirms the notion that this domain plays a major role in nNOS protein quality control.

As shown in Fig. 7A, the location of the ubiquitination sites were mapped onto a crystal structure of the nNOS oxygenase domain (27), using Pymol (DeLano Scientific). The crystal structure contains a dimer of two oxygenase domain monomers, one shown in blue and one in gray. The heme is shown in the active site cleft in red. Two of the lysine residues that were identified as ubiquitination sites, for one monomer, are shown in yellow (K370 and K386). These residues on the other monomer are on the back of the structure. The ubiquitination sites prior to residue 297 (K24, K33, K143, K188, K225, K229, K245) are not included on the crystal structure, however, the N-terminus of the oxygenase domain is notated to depict where these sites would likely be located (N_1). The C-terminus is also indicated (C_2), which is where the calmodulin-binding domain would begin. The N- and C-termini for the opposing monomers are located on the back of the structure.

To more fully appreciate the location of the ubiquitination sites, a full-length structure is needed. There is no full-length structure of nNOS, but Garcin *et al.* (28) have utilized known biochemical data and studies on the flow of electrons through the enzyme to assemble the structures of its components, to depict the full structure with the reductase (*Red*) domain binding below the oxygenase (*Oxy*) domain, as we have adapted in Fig. 7B. The calmodulin-binding region is also not included in the crystal structure, but it is believed to be located in the space between the oxygenase and reductase domains as indicated in Fig. 7B (*CaM*), based on the location of the C-terminus (*C*) of the

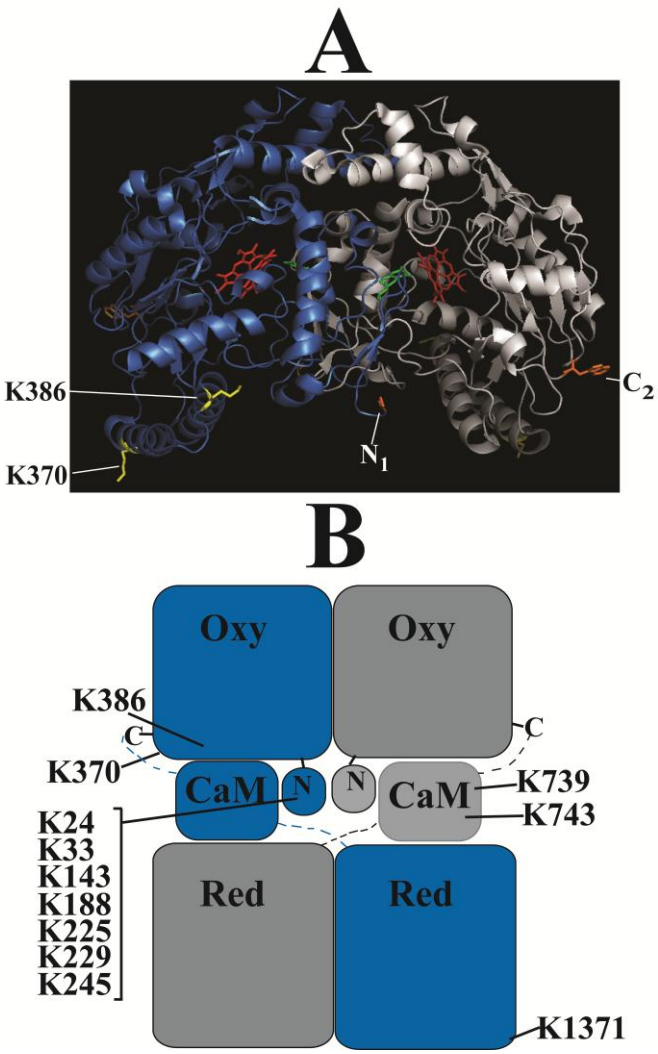

Figure 4.7. Schematic of the ubiquitination sites on the structure of nNOS. *A,* nNOS ubiquitination sites depicted on the crystal structure of the oxygenase domain. The nNOS oxygenase domain (residues 297 through 716) is shown, with one monomer in blue and one in gray. Only two of the 12 ubiquitination sites are included in this structure as indicated (K370, K386) and the lysine residues are shown in yellow. Seven of the ubiquitination sites are on the N-terminal residues, which are not present in the crystal structure. Two of the ubiquitination sites are in the calmodulin binding domain, which is also not present in the crystal structure. The N-terminus (N_1) of one monomer and the C-terminus (C_2) of the other monomer are shown in orange where indicated. The opposing N- and C-termini are located on the back of the structure. *B,* 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.* 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 dotted line. All 12 ubiquitination sites are indicated. The N-termini (N) and C-termini (C) of the oxygenase domains are indicated. Statistical probability is expressed as follows: *, $p<0.05$.

oxygenase domain and the N-terminus of the reductase domain and the limited flexibility of this region (28). The lysine residues corresponding to the identified ubiquitination sites are indicated where they would be located on the structure. According to this schematic drawing, it appears that the ubiquitination sites are primarily located at the interface between the oxygenase domain and where the reductase and the calmodulin-binding domains are believed to bind (28). It is of interest that these ubiquitination sites are located near and in the calmodulin-binding domain. Previous studies have demonstrated the ability of calmodulin, in the presence of Hsp90, to block nNOS ubiquitination (15).

As an initial attempt to determine the functional significance of the ubiquitination sites, we created a mutant (nNOS 7R) that has all seven lysines that occur in the calmodulin-binding domain of nNOS mutated to arginines, which cannot be conjugated to ubiquitin. Arginine was chosen because it will maintain the charge of the residue, and it should not significantly alter the structure or folding of the enzyme, and the nNOS 7R mutant found it to be active. The *in vitro* mono-ubiquitination of the nNOS 7R mutant was similar to the wildtype enzyme, which indicates that the other ubiquitination sites could be accounting for the majority of the mono-ubiquitination of nNOS. The *in vitro* poly-ubiquitination as well as the degradation of the nNOS 7R mutant are significantly decreased, indicating that this region greatly influences the protein quality control of nNOS. Studies have shown that Hsp90 and calmodulin enhance the binding of each other to both nNOS and endothelial NOS (29, 30). Additionally, it has been proposed that Hsp90 acts to stabilize an open state of the ligand-binding cleft, in order to facilitate substrate access or to prevent further cleft unfolding that triggers Hsp70-dependent

ubiqtuitination (15, 31). Moreover, inhibition of Hsp90 has been shown to disrupt the binding of Hsp90 with its client proteins, such as GR (glucocorticoid receptor) and a mutant p53 that is more stable than wildtype p53, allowing for ubiquitination by CHIP and subsequent degradation of these proteins (18, 32). Disruption of Hsp90 binding could decrease the ability of calmodulin to bind, thus making the calmodulin-binding region available for ubiquitination. Thus, in a situation in which calmodulin and Hsp90 are not present, Hsp70/CHIP-mediated ubiquitination can occur in the oxygenase and calmodulin-binding domains.

Different types of poly-ubiquitin chains have been shown to play a role in degradation as well as signaling processes. K48-linked chains are widely accepted as a target for proteasomal degradation (33), whereas non-canonical K63-linked chains play a role in DNA repair and signal transduction (34). Recent studies, however, reveal that all poly-ubiquitin chains may target proteins for degradation (35, 36). We found that poly-ubiquitination of nNOS can occur through any of the lysines on ubiquitin. These data are consistent with previous reports that when CHIP is paired with the E2 UbcH5a, it can form poly-ubiquitin chains with any of the individual ubiquitin lysine mutants (37). Thus, the poly-ubiquitination of nNOS can occur through any of the ubiquitin lysines, and any of these chains could lead to the proteasomal degradation or molecular signaling of nNOS. In contrast, pairing CHIP with the E2 UbcH13/Uev1a leads poly-ubiquitination of Troponin I with K63 chains exclusively (38). Thus, it is possible that other E2 enzymes may confer selectivity to nNOS ubiquitination by CHIP.

Given the fact that NO is a very short-lived molecule that cannot be stored or released from vesicles, the steady state levels of NO are due primarily to the levels of

active NOS. Thus, it is important to understand the molecular mechanisms by which nNOS is regulated. It is of particular interest due to the fact that drugs and other xenobiotics can significantly impact the stability of nNOS, whether it is by suicide-inactivation leading to destabilization, or by N^G-nitro-L-arginine binding leading to stabilization. Here we show the first evidence of the sites of ubiquitination of nNOS, which will aid in ultimately understanding where chaperones bind nNOS for ubiquitination and targeting for degradation.

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