Modulation of Heme/Substrate Binding Cleft of Neuronal Nitric-oxide Synthase (nNOS) Regulates Binding of Hsp90 and Hsp70 Proteins and nNOS Ubiquitination*

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Background: Hsp90 and Hsp70 act together as machinery for protein quality control.

Results: Both chaperones sense conformational changes (opening/closing) in ligand binding clefts.

Conclusion: Hsp90 inhibits substrate ubiquitination and degradation, whereas Hsp70 promotes ubiquitination and degradation.

Significance: The opposing effects of the two chaperones can account for the triage of damaged and aberrant proteins.

Like other nitric-oxide synthase (NOS) enzymes, neuronal NOS (nNOS) turnover and activity are regulated by the Hsp90/Hsp70-based chaperone machinery, which regulates signaling proteins by modulating ligand binding clefts (Pratt, W. B., Morishima, Y., and Osawa, Y. (2008) J. Biol. Chem. 283, 22885–22889). We have previously shown that nNOS turnover is due to Hsp70/CHIP-dependent ubiquitination and proteasomal degradation. In this work, we use an intracellular cross-linking approach to study both chaperone binding and nNOS ubiquitination in intact HEK293 cells. Treatment of cells with N\(^{\text{G}-}\)nitro-L-arginine, a slowly reversible competitive inhibitor that stabilizes nNOS, decreases both nNOS ubiquitination and binding of Hsp90, Hsp70, and CHIP. Treatment with the calcium ionophore A23187, which increases Ca\(^{2+}\)-calmodulin binding to nNOS, increases nNOS ubiquitination and binding of Hsp90, Hsp70, and CHIP in a manner that is specific for changes in the heme/substrate binding cleft. Both Hsp90 and Hsp70 are bound to the expressed nNOS oxygenase domain, which contains the heme/substrate binding cleft, but not to the reductase domain, and binding is increased to an expressed fragment containing both the oxygenase domain and the calmodulin binding site. Overexpression of Hsp70 promotes nNOS ubiquitination and decreases nNOS protein, and overexpression of Hsp90 inhibits nNOS ubiquitination and increases nNOS protein, showing the opposing effects of the two chaperones as they participate in nNOS quality control in the cell. These observations support the notion that changes in the state of the heme/substrate binding cleft affect chaperone binding and thus nNOS ubiquitination.

The function and turnover of a wide variety of signaling proteins are regulated by Hsp90\(^3\) (reviewed in Ref. 1). The Hsp90/Hsp70-based chaperone machinery regulates signaling proteins by modulating ligand binding clefts (reviewed in Ref. 2), and these proteins constantly undergo cycles of Hsp90 heterocomplex assembly and disassembly in the cytoplasm and nucleoplasm (1). Two types of cycling with Hsp90 occur. The classical Hsp90 “client” proteins, such as steroid receptors and many protein kinases, form Hsp90 heterocomplexes that are stable enough to be isolated and analyzed biochemically. We call this “stable cycling” with Hsp90, and the turnover of these proteins is stringently regulated by the chaperone (2). Formation of heterocomplexes with Hsp90 inhibits client protein turnover, and treatment with an Hsp90 inhibitor, such as geldanamycin, uniformly triggers client protein degradation (3). Other signaling proteins, such as the nitric-oxide synthase (NOS) enzymes, 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 turnover of these proteins is not as affected by Hsp90 inhibitors as the classical client proteins (2). Degradation of both types of 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 (4) and parkin (5).

Ligand binding clefts are hydrophobic clefts that must be open to allow access of ligands, such as steroids or ATP, to their binding sites within the interior of the protein. In the absence of the chaperone machinery, ligand binding clefts are dynamic, shifting to varying extents between the closed and open states. When clefts open, hydrophobic residues of the interior of the protein are exposed to solvent, and continued opening may progress to protein unfolding. Therefore, the extent to which the ligand binding cleft is open determines ligand access and thus protein function, but clefts are inherent sites of conformational instability. We have proposed that the stability of the open state of the cleft is modulated by the Hsp90/Hsp70-based chaperone machinery (2, 6), and in this study, we further develop that model.

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3 The abbreviations used are: Hsp, heat shock protein; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; CHIP, carboxyl terminus of Hsp70-interacting protein; E3, ubiquitin-protein isopeptide ligase; CaM, Ca\(^{2+}\)-calmodulin; NNA, N\(^{\text{G}-}\)nitro-L-arginine; DSP, dithiobis(succinimidylpropionate).
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The NOS enzymes, including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), are signaling proteins whose activity is enhanced by Hsp90 (7–14). These enzymes are cytochrome P450-like hemoproteins that catalyze the conversion of l-arginine to nitric oxide and citrulline by a process that requires NADPH and molecular oxygen (15). NOS enzymes are bidomain in structure with an oxygenase domain, which contains the binding sites for heme, substrate, and tetrahydrobiopterin, and a reductase domain, which contains the binding sites for FMN, FAD, and NADPH. NOS enzymes are highly regulated, requiring homodimerization and binding of Ca$^{2+}$-calmodulin (CaM) for activity, 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 binding of each other to both eNOS and nNOS (11, 12, 14, 16). Another mechanism for regulation is the selective ubiquitin-dependent proteasomal degradation of dysfunctional NOS (reviewed in Ref. 17).

Because nNOS is cytosolic and because metabolism-based inactivators, such as $N^G$-amino-$\gamma$-arginine and the antihyperensive drug guanabenz, cause covalent alteration in the heme/substrate binding cleft that triggers nNOS ubiquitination and proteasomal degradation (18, 19), we have found the enzyme to be a good model for studying how the state of the ligand binding cleft affects ubiquitination. For example, guanabenz treatment leads to the oxidation of tetrahydrobiopterin with formation of a pterin-depleted cleft that triggers nNOS ubiquitination and proteasomal degradation (18, 20, 21). A number of other inhibitors, such as $N^G$-amino-$\gamma$-arginine, cross-link heme to the enzyme (17, 22), a modification that was shown in a myoglobin model to cause opening of the heme/substrate binding cleft (23) to yield a more unfolded state of the protein (24) that triggers ubiquitination. Both geldanamycin and $N^G$-amino-$\gamma$-arginine promote nNOS ubiquitination by a purified CHIP-dependent ubiquitinating system (25).

As with eNOS and iNOS, treatment of cells with an Hsp90 inhibitor leads to nNOS degradation via the ubiquitin-proteasome pathway (8, 18). We have shown that both CHIP and parkin can function as E3 ligases for nNOS ubiquitination (5, 26) but that CHIP accounts for all of the nNOS ubiquitinating activity (27) in the reticulocyte lysate ubiquitination system of Hershko et al. (28). Ubiquitination of nNOS by a purified, CHIP-dependent ubiquitinating system (25, 26) and by the reticulocyte lysate system (27, 29) is dependent upon Hsp70. In contrast to Hsp70, which stimulates nNOS ubiquitination when added to the purified CHIP-dependent system, Hsp90 inhibits ubiquitination (25). Like the stimulation of nNOS activity by Hsp90, inhibition of nNOS ubiquitination by Hsp90 is calmodulin-dependent (25), suggesting that both activation and stabilization result from the same interaction of Hsp90 with the enzyme.

In this work, we will use the ligand $N^G$-nitro-$\gamma$-arginine (NNA), a slowly reversible, competitive inhibitor of nNOS that stabilizes the enzyme (30), to modulate the binding of Hsp90 and Hsp70 to nNOS in HEK293 cells. We also use the calcium ionophore A23187 to modulate chaperone binding through CalM binding. Inasmuch as CalM enhances electron flux from flavin bound to the reductase domain to heme bound within the cleft (31), CalM binding is likely to affect the state of the cleft. To obtain mechanism-based inactivation of nNOS, cells must be treated with calcium ionophore to activate the enzyme. As we show here, treatment with calcium ionophore markedly changes the binding of Hsp90 and Hsp70 to nNOS in HEK293 cells, and because of this, we have not been able to tease out the effects of $N^G$-amino-$\gamma$-arginine and guanabenz on chaperone binding.

Because Hsp90 cycles dynamically with the holo-nNOS homodimer, we have used an intracellular cross-linker to trap nNOS-chaperone complexes that can then be immunoprecipitated from cell lysates with anti-nNOS. We show that treatment of HEK293 cells with the calcium ionophore A23187 increases nNOS ubiquitination and the amount of Hsp90, Hsp70, and CHIP in nNOS heterocomplexes in a manner that is specific for changes in the heme/substrate binding cleft. In contrast, the stabilizing ligand NNA decreases both nNOS ubiquitination and the recovery of Hsp90, Hsp70, and CHIP in nNOS heterocomplexes. Both Hsp90 and Hsp70 are bound to the expressed nNOS oxygenase domain but not to the reductase domain, and binding is increased to an expressed fragment containing both the oxygenase domain and the CalM binding site. Overexpression of Hsp70 promotes and overexpression of Hsp90 inhibits nNOS ubiquitination, with Hsp70 decreasing and Hsp90 increasing nNOS protein levels in HEK293 cells. This confirms that the two chaperones have opposing effects as they participate together in nNOS quality control. Taken together, the demonstration that ligand-dependent changes to the heme/substrate binding cleft regulate chaperone binding in the cell and that the chaperones bind to the oxygenase domain, which contains the heme/substrate binding cleft, strongly support the general model where the Hsp90/Hsp70-based chaperone machinery regulates signaling proteins by modulating ligand binding clefts (2).

**EXPERIMENTAL PROCEDURES**

**Materials**

Protein A-Sepharose, radicicol, pifithrin-$\mu$, calcium ionophore A23187, $L$- and $D$-isomers of $N^G$-nitro-$\gamma$-arginine ($L$-NNA, $D$-NNA), rabbit polyclonal anti-nNOS (raised against residues 251–270), and mouse anti-$\beta$-tubulin were purchased from Sigma. $N^G$-amino-$\gamma$-arginine, mouse anti-Hsp90 monoclonal antibody (AC88), mouse anti-Hsp70/Hsc70 monoclonal antibody (N27F3-4), and lactacystin were from Enzo Life Sciences (Plymouth Meeting, PA). Rabbit anti-CHIP antibody was from Affinity BioReagents (Golden, CO). Small interfering RNAs (siRNAs) for Hsp90, Hsc70, and Hsp70, the K19 rabbit polyclonal antibody specific for Hsc70, and anti-HA antibody-conjugated agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal IgG C92F3A-5 specific for Hsp70 was from Assay Designs (Ann Arbor, MI). Minimum essential medium, Dulbecco’s modified Eagle’s medium, and G418 (Geneticin®) were from Invitrogen. The cDNA for rat nNOS and HEK293 cells stably transfected with rat nNOS were provided by Dr. Solomon Snyder (Johns Hopkins Medical School, Baltimore, MD). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (University of Michigan, Ann Arbor,
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MI), and human CHIP cDNA was from Dr. Cam Patterson (University of North Carolina, Chapel Hill, NC). The intracellular cross-linker dithiobis(succinimidylpropionate) (DSP) was from Thermo Scientific. Restriction endonucleases and modifying enzymes were from New England Biolabs (Beverly, MA). All oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA).

Methods

Cell Culture and Transient Transfection—HEK293 cells stably transfected with rat nNOS were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone®), 20 mM Hepes, pH 7.4, and G418 (0.5 mg/ml) as described previously (32). Twenty hours before harvesting, the cells were cultured in DMEM containing 0.1 mM arginine (low arginine DMEM). The HEK293T cells used for transient transfection were obtained from the American Type Culture Collection and cultured in minimum essential medium supplemented with 10% fetal bovine serum. Transient transfections of 293T cells were carried out with the use of a standard calcium phosphate method in 10-cm dishes. A solution (100 μl) of 2.5 mM CaCl2 in 10 mM Hepes, pH 7.2, and the desired amount of plasmid DNA was diluted to 1.0 ml with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.3), and 1 volume of this 2× Ca/DNA solution was added dropwise to an equal volume of Hepes-buffered saline solution (275 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 10 mM dextrose, 40 mM Hepes, pH 7.2). The two solutions were mixed and added to the cell culture medium after 15 min of incubation. Cells at 70–80% confluence were transfected with cDNAs for His-HA-ubiquitin (3 μg), CHIP (1 μg), nNOS (3 μg), and Hsp70 (3 μg) or Hsp90 (3 μg) with the total amount of cDNA being kept constant with vector plasmid. The transfection efficiency of HEK293T cells was in the 60–80% range. In this protocol, 10 μM lactacystin is present for 18 h before cell lysis at 48 h. For experiments depleting Hsp90 or Hsp70, HEK293 cells stably transfected with nNOS were seeded at 3 × 105 cells/well in 6-well plates 1 day before the addition of siRNA in Lipofectamine RNAiMAX transfection reagent from Invitrogen. Forty-eight hours later, cells were harvested in SDS sample buffer, boiled, and electrophoresed.

Plasmids—The rat nNOS cDNA was subcloned from PVL1393 (8) into the EcoRI and NotI sites of pcDNA3.1+. Expression plasmids for the isolated oxygenase domain (Oxy-(1–720)), the oxygenase domain containing the CaM binding site (OxyCaM-(1–756)), and the isolated reductase domain (Red-(746–1429)) of nNOS were created by inserting the corresponding PCR fragments into mammalian expression vector pcDNA3.1+. The HA tag was attached to the C terminus of the Oxy and OxyCaM domains (designated Oxy-HA and OxyCaM-HA, respectively), and an HA tag was attached to the N terminus of the reductase domain (designated HA-Red). The coding sequence of human Hsp70 and Hsp90 was amplified by PCR and subcloned into pcDNA4/HisMax for expression in mammalian cells. The inserts of all recombinant plasmids were sequenced to ensure accuracy.

Chemical Cross-linking and Immunoprecipitations—nNOS-expressing HEK293 cells were treated with 200 μM NNA or 4 μM calcium ionophore (A 23187) for 30 min at 37 °C. After incubation, cells were washed twice with room temperature phosphate-buffered saline (PBS) and then resuspended in cross-linking buffer that contains 1.5 mM DSP and protease inhibitor in PBS. The cells were incubated at room temperature for 30 min with rotation, and the cross-linking was arrested by the addition of Tris, pH 7.5, at a final concentration of 20 mM for 15 min at 4 °C. Cells were subsequently resuspended in 0.1 ml of buffer A (0.3% Triton X-100, 0.7% n-octylglucoside in PBS) and sonicated twice for 25 s with a 30-s interval. Whole-cell lysates were obtained by centrifugation at 14,000 g for 20 min at 4 °C to remove cellular debris. Equal amounts of proteins (~400 μg) from HEK293 cells lysates were incubated with 20 μl of anti-nNOS antibody preconjugated with 140 μl of protein A-Sepharose in a total volume of 300 μl of buffer B (0.1% Triton X-100, 0.2% n-octylglucoside in PBS) for 4 h at 4 °C with agitation. Immunoprecipitates were washed two times with buffer B and three times with PBS by resuspension and centrifugation (3000 × g, 2 min each). Fifty microliters of SDS sample buffer (5% SDS, 20% glycerol, 6 mg/ml dithiothreitol, and 0.02% broman phenol blue in 125 mM Tris-HCl, pH 6.8) were added to each sample tube, and tubes were heated at 100 °C for 5 min to extract the bound target proteins. In studies where HA was immunoadsorbed, 25 μl of anti-HA antibody-conjugated agarose replaced anti-nNOS IgG and protein A-Sepharose. For ubiquitination studies, the cell pellet was directly homogenized in HS buffer (10 mM Hepes, pH 7.4, 0.32M sucrose, 2 mM EDTA, 6 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 15 mM sodium vanadate, 1% Nonidet P-40, and 5 mM N-ethylmaleimide) without cross-linking.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—After removing the protein A-Sepharose by a brief centrifugation at room temperature, the supernatant was resolved by SDS-PAGE under reducing conditions. Western blot was performed with a PVDF membrane and probed with anti-Hsp90, Hsp70, CHIP, or nNOS antibody as indicated. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (ECL) and X-Omat film. The mono-ubiquitinated conjugate is the predominant ubiquitinated nNOS species detected in HEK293 cells (19). Thus, the mono-ubiquitinated nNOS bands were scanned, and the relative densities were determined with the ImageJ software (rsb.info.nih.gov/ij/). nNOS bands were exposed for a short time to visualize non-ubiquitinated nNOS and for a long time to visualize the mono-ubiquitinated nNOS bands. To correct for any minor differences in nNOS protein in different immunoprecipitates, mono-ubiquitinated nNOS bands were normalized according to the addition of the non-ubiquitinated nNOS. Relative densities for bands were normalized according to the short time exposure of the non-ubiquitinated nNOS. Relative densities for at least three experiments are presented in bar graphs as the percentage of the condition with the greatest ubiquitination ± S.E. Significance of difference was determined by one-way analysis of variance (Tukey’s multiple comparison test). Statistical probability is expressed as *, p < 0.05, **, p < 0.01, ***, p < 0.001 in Figs. 2–8.
RESULTS

Intracellular Cross-linking Is Required for Detection of nNOS-Chaperone Complexes—In the experiment of Fig. 1, HEK293 cells stably expressing nNOS were either untreated or treated for 30 min with the nNOS stabilizer NNA. Cells for each condition were then exposed to DSP or vehicle, and cell lysates were prepared and immunoprecipitated for nNOS. Hsp90 and Hsp70 were detected in nNOS complexes immunoadsorbed from cells treated with the cross-linker (lanes 1 and 2) but not in immunoprecipitates from cells not exposed to DSP (lanes 3 and 4). Thus, in HEK293 cells, the intracellular cross-linker is required to visualize the chaperones interacting with nNOS. It also appears that treatment of cells with NNA decreased the amount of the chaperones and CHIP co-immunoprecipitating with nNOS.

Modulation of Chaperone Binding by Treatment with NNA or Calcium Ionophore—Fig. 2 shows that treatment of cells with the nNOS stabilizer NNA (lane 3) does indeed decrease the amount of Hsp90, Hsp70, and CHIP co-immunoprecipitating with nNOS, and NNA also decreases the level of nNOS ubiquitination when compared with untreated cells (lane 2). The chaperones are not present in immunoprecipitates prepared from HEK293 cells that are not expressing nNOS (lane 1), confirming their presence in an nNOS-specific heterocomplex. The ubiquitination observed in untreated cells (lane 2) reflects a basal level of nNOS autoinactivation occurring in the absence of a rise in intracellular Ca\(^{2+}\) concentration. When the Ca\(^{2+}\) concentration is increased by treating cells with the calcium ionophore A23187 (lane 4), both the level of nNOS ubiquitination and the amounts of Hsp90, Hsp70, and CHIP co-immunoprecipitating with nNOS increase. The higher level of ubiquitination likely reflects increased autoinactivation occurring when nNOS enzymatic activity is stimulated by CaM binding. The increased ubiquitination may reflect increased binding of Hsp70 and CHIP to nNOS that is unfolding in response to autoinactivation by active oxygen species generated within the heme/substrate binding cleft. The increased binding of Hsp90 in the presence of the ionophore is probably due to the fact that CaM and Hsp90 increase the binding of each other to nNOS (14, 16).

To determine whether the ionophore effects on chaperone binding reflect changes within the heme/substrate binding cleft, we used the slowly reversible inhibitor L-NNA or its inactive isomer D-NNA to demonstrate stereospecific modulation of the ionophore effect. As shown in Fig. 3, L-NNA (lane 3) protects nNOS from the increases in ubiquitination and chaperone binding that are seen with the ionophore (lane 2), whereas D-NNA (lane 4) does not protect. This stereospecific protection by ligand binding within the heme/substrate binding cleft supports the notion that the state of the cleft affects chaperone binding.

Binding of Chaperones to Expressed nNOS Domains—The heme/substrate binding cleft is located in the oxygenase domain, and the experiments shown in Fig. 4 show that the oxygenase domain alone is sufficient for binding of Hsp90 and Hsp70. In Fig. 4A, HA-tagged oxygenase domain or oxygenase domain plus the CaM binding segment or the reductase domain were expressed, cross-linked, and immunoprecipitated with anti-HA antibody. Hsp90 and Hsp70 co-immunoprecipitated with the oxygenase domain (lane 1) and the oxygenase domain containing the CaM binding site (lane 2) but not with the reductase domain (lane 3). In Fig. 4B, full-length nNOS, the oxygenase domain, or the oxygenase domain plus the CaM binding segment were expressed, cross-linked, and immuno-
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precipitated with anti-nNOS. Hsp90 and Hsp70 co-immunoprecipitated with all three, and as in Fig. 4A, more of each chaperone appeared to be present with the OxyCaM than with the Oxy domain alone. To correct for possible differences in the recovery of domains, the bands from several experiments like that of Fig. 4A were scanned, normalized by the HA band, and plotted in the bar graphs of Fig. 4C. Although the oxygenase band is sufficient for binding of Hsp90 and Hsp70, the presence of the CaM binding segment increases recovery. This suggests that the affinity of chaperone binding may be higher when the CaM binding segment is present. This would be consistent with reports that purified CaM and Hsp90 increase binding of each other to eNOS and nNOS (11, 12, 14, 16).

Opposing Effects of Hsp90 and Hsp70 on nNOS Ubiquitination in HEK Cells—We have shown previously that nNOS ubiquitination by a purified CHIP-containing ubiquitinating system is promoted by Hsp70 and inhibited by Hsp90 (25). To determine whether the two chaperones have opposing actions on nNOS ubiquitination in the cell, each of them was overexpressed in HEK293T cells along with nNOS in a protocol we have used previously to demonstrate CHIP-dependent nNOS ubiquitination under transient transfection conditions (26). Fig. 5A shows the levels of Hsp90 and Hsp70 in lysates of HEK293T cells 48 h after transient transfection with cDNA for each chaperone in addition to cDNAs for nNOS, CHIP, and His-HA-ubiquitin. Fig. 5B shows the effect of overexpression of Hsp70 (lane 2) or Hsp90 (lane 3) on nNOS mono-ubiquitination. The data from scans of three experiments are shown in the bar graphs. The region of the gel above the mono-ubiquitinated nNOS was blotted with anti-HA antibody to show the polyubiquitinated nNOS, which underwent the same changes with Hsp70 or Hsp90 overexpression. As was found in the purified ubiquitinating system (25), overexpression of Hsp70 promoted and overexpression of Hsp90 inhibited nNOS ubiquitination in HEK cells.

To determine whether the opposing effects of Hsp90 and Hsp70 on nNOS ubiquitination are reflected by the appropriate opposing effects on nNOS protein level, HEK293T cells were transfected with nNOS cDNA and increasing amounts of Hsp90 or Hsp70 cDNA. Fig. 6 shows the levels of nNOS in lysates of HEK293T cells 48 h after transient transfection with cDNA for Hsp90 (Fig. 6A) or Hsp70 (Fig. 6B) in addition to cDNAs for nNOS and CHIP. Overexpression of Hsp90 yields an increase in nNOS protein, and overexpression of Hsp70 yields a decrease in nNOS protein, consistent with the inhibition and promotion of ubiquitination, respectively, shown in Fig. 5B. In Fig. 6C, HEK293 cells stably expressing nNOS were transfected with siRNA for human Hsp90α/β, Hsc70, or Hsp70. Decreasing the level of Hsp90 (lane 3) caused a decrease in nNOS, consistent with a decrease in the stabilizing effect of Hsp90 shown in Fig. 6A. HEK293 cells stably expressing nNOS are stressed, probably as a result of the generation of active oxygen species by the enzyme, and they have very high levels of Hsp70 with respect to Hsc70 (Fig. 6C). Decreasing the level of Hsc70 (Fig. 6C, lane 4) does not affect the level of nNOS or of Hsp70. In these cells, the Hsp70 level can be decreased only by about one-half (lane 5), which is not sufficient to affect the level of nNOS. Transfection with siRNA for both Hsc70 and Hsp70 still leaves half of the Hsp70, and again, the level of nNOS is unaffected (lane 6).

In that overexpression of the chaperones altered the nNOS mono-ubiquitination in Fig. 5B, we asked whether inhibition of each chaperone had the opposite effect on nNOS ubiquitination. Radicicol is an inhibitor that binds to the N-terminal domain of Hsp90, and treatment with radicicol depletes cells of classical Hsp90 clients, such as Raf-1 and mutant p53 (33). In Fig. 7A, HEK293 cells stably transfected with nNOS were treated with 20 μM radicicol, a concentration of the inhibitor we have previously shown to prevent heme binding and activation of apo-nNOS in insect cells (34). As shown in Fig. 7A, treatment with radicicol (lane 3) increases nNOS mono-ubiquitination when compared with the level seen without treatment (lane 1). When cells are treated with both the calcium ionophore and radicicol (lane 4), ubiquitination is greater than that seen with the ionophore alone (lane 2). In Fig. 7B, cells were treated with pifithrin-μ, a small molecule inhibitor of Hsp70 (35). Pifithrin-μ inhibited nNOS ubiquitination in the absence (cf. lanes 1 and 3) or presence (cf. lanes 2 and 4) of calcium ionophore. This is consistent with cell-free observations where ubiquitination of nNOS by a purified, CHIP-dependent ubiquitinating system (25, 26) and by the reticulocyte lysate system (27, 29) is dependent upon Hsp70. As shown in Fig. 7C, treatment with the inhibitors did not affect the level of total Hsp90 or Hsp70 in the HEK cells.
Treatment of cells with the inhibitors radicicol and geldanamycin has been shown to reduce Hsp90 binding to a wide variety of Hsp90 target proteins (1), including all three NOS enzymes (10, 12, 36). In contrast, pifithrin-μ has not been widely studied, although it has been shown to interact specifically with Hsp70 and not to interact with Hsp90, and treatment of cells with pifithrin-μ has been shown to reduce Hsp70 binding to p53 (35). In Fig. 7D, nNOS was immunoprecipitated from cytosol prepared from HEK293 cells treated with radicicol or pifithrin-μ, and nNOS-associated Hsp90 and Hsp70 were visualized by immunoblotting. Radicicol reduces the binding of Hsp90 but not Hsp70, and pifithrin-μ reduces the binding of Hsp70 but not Hsp90.

In Fig. 8, the effects of radicicol and pifithrin-μ on nNOS ubiquitination were compared in the absence and presence of overexpression of Hsp90 or Hsp70. When Hsp90 is overexpressed, radicicol yields roughly the same increase in ubiquitination relative to the untreated control as in the absence of overexpression (Fig. 8A). Similarly, pifithrin-μ yields roughly the same inhibition of ubiquitination in the presence and absence of Hsp70 overexpression (Fig. 8B). Because Hsp90 and Hsp70 are highly abundant proteins, their overexpression only doubles the amount of each chaperone (Fig. 5A). Thus, in each case, we only reduce the ratio of inhibitor to chaperone by about one-half when each chaperone is overexpressed, yielding roughly the same change in ubiquitination with and without overexpression. Taken together, the data of Figs. 5, 7, and 8 support the notion that Hsp90 and Hsp70 have opposing effects, with Hsp90 inhibiting and Hsp70 promoting nNOS ubiquitination.

**DISCUSSION**

The oxygenase domain of nNOS contains the heme/substrate binding cleft and is the domain that interacts with Hsp90 (Fig. 4). Both pulldown and peptide competition experiments suggest that a region (amino acids 300–400) in the oxygenase domain of endothelial NOS is important for Hsp90 binding (37, 38). Here, we show that the oxygenase domain of nNOS is also the site of its interaction with Hsp70. The finding that Hsp90 and Hsp70 bind to domains containing ligand binding clefts also pertains to signaling proteins that are classic Hsp90 clients. For example, both chaperones interact with the ligand binding domains of the steroid receptors (reviewed in Ref. 39). Also, Hsp90 has been shown to interact with the catalytic domains, which contain the ATP binding clefts, of protein kinase clients, such as v-Raf (40) and ErbB-2 (41).

Although it has been demonstrated that Hsp90 interacts with domains containing ligand binding clefts, there is no common specific binding motif defining a surface for interaction, and our notion is that the chaperone binds at the opening where hydrophobic ligand binding clefts merge with the protein surface (1, 2). Such cleft openings are a topological feature of virtually all proteins in native conformation, raising the possibility that Hsp90 interacts with a much broader range of proteins than the...
limited subset of stable cycling, stringently regulated proteins that have been the focus of interest as Hsp90 clients. The use of cross-linking techniques, such as that employed here with nNOS, may identify multiple proteins that cycle dynamically with Hsp90 and are regulated in less dramatic fashion than the classic Hsp90 clients. It is not known what feature of a ligand binding cleft (e.g. dynamics of opening/closing?) determines whether a protein will undergo stable or dynamic cycling with Hsp90. However, there are several examples where mutations within the ligand binding domain of steroid receptors and catalytic domains of protein kinases convert these classic Hsp90 clients to the dynamic cycling that is seen with the NOS enzymes (2).

Binding of the slowly reversible inhibitor NNA within the heme/substrate binding site of nNOS decreases binding of Hsp90 and Hsp70 to nNOS and decreases nNOS ubiquitination (Fig. 2). The ability of ligand binding to modulate Hsp90 binding was originally reported for steroid receptors (39), and steroid-dependent dissociation of Hsp90 is often presented in textbook models as the first step in steroid hormone action. It is now realized that binding of steroid within the cleft promotes a temperature-dependent collapse of the cleft to the closed state, converting the receptor from stable Hsp90 cycling to dynamic Hsp90 cycling (2). A study of Hsp90 binding to iNOS suggests that the binding of heme to the apo-iNOS monomer may drive a similar conversion from stable to dynamic cycling (42). Heme binding to apo-NOS drives its homodimerization to the active holo-NOS enzyme, and heme insertion into apo-nNOS requires Hsp90. Stuehr and co-workers (42) have shown that apo-iNOS forms stable complexes with Hsp90, whereas heme-bound holo-iNOS does not, consistent with conversion from stable to dynamic cycling. Similarly, binding of NNA may favor a more closed conformation of the heme/substrate binding cleft of holo-nNOS to favor even more dynamic cycling with Hsp90 and decreased capture of the nNOS-Hsp90 heterocomplex upon cross-linking. CaM binding is required for nNOS to be active, and CaM binding may favor a more open state of the ligand binding cleft that cycles less dynamically with Hsp90, increasing capture of the nNOS-Hsp90 heterocomplex upon cross-linking (Fig. 2).

To our knowledge, there have been no studies of ligand effects on Hsp70 recovery with steroid receptors, but NNA binding to nNOS decreases the recovery of both Hsp70 and CHIP (Fig. 2). Again, NNA binding may favor a more closed

![FIGURE 5. Hsp70 increases and Hsp90 inhibits nNOS ubiquitination in HEK293T cells.](image)

**A**, overexpression of Hsp90 and Hsp70. Cells were transfected with cDNAs for His-HA-ubiquitin, CHiP, nNOS, and Hsp90 or Hsp70 or vector plasmid. After 48 h, lysates were prepared and Western blotted for Hsp70, Hsp90, and β-tubulin. **B**, effect of Hsp90 and Hsp70 overexpression on nNOS mono-ubiquitination (mono-Ub). Cells were transfected as above, and lysates were immunoprecipitated with anti-nNOS antibody and Western blotted with anti-nNOS and anti-HA. The upper panel shows the region above mono-ubiquitinated nNOS blotted with anti-HA antibody. The lower panel shows a short exposure for unmodified nNOS protein, and the middle panel shows a long exposure for the mono-ubiquitinated nNOS. The bar graphs show the relative densities of mono-ubiquitinated nNOS bands expressed as means ± S.E. for three separate experiments. ***, p < 0.001, *, p < 0.05. HA-Ub, HA-ubiquitin.**

![FIGURE 6. Hsp90 increases and Hsp70 decreases level of nNOS protein in HEK293 cells.](image)

**A**, overexpression of Hsp90. HEK293T cells were transfected with cDNAs for CHIP, nNOS, and increasing amounts of Hsp90 as noted above the immunoblot lanes. After 48 h, cell lysates were prepared in SDS sample buffer and aliquots were electrophoresed and Western blotted for nNOS, Hsp90, Hsp70, and β-tubulin. **B**, overexpression of Hsp70. Protocol was as above except for cDNA for Hsp70 instead of Hsp90. **C**, decreasing expression of Hsp90, Hsc70, and Hsp70. HEK293 cells stably expressing nNOS were transfected with scrambled siRNA (lane 1) or siRNA for GAPDH (lane 2), Hsp90u/β (lane 3), Hsc70 (lane 4), Hsp70 (lane 5), or both Hsc70 and Hsp70 (lane 6). After 48 h, cell lysates were prepared and Western blotted for nNOS, Hsp90, and GAPDH and with antibodies specific for Hsc70 or Hsp70. The bar graphs show the relative densities of the nNOS bands expressed as the means ± S.E. for three separate experiments. ***, p < 0.001.**
Regulation of nNOS Chaperone Binding and Ubiquitination

In a broader sense, the possibility that ligands affect Hsp70 binding by modulating cleft conformation could contribute to a major way to explaining how substrates and inhibitors stabilize enzymes in general.

We have proposed that the Hsp90/Hsp70-based chaperone machinery may be the major mechanism for quality control of damaged proteins via the ubiquitin-proteasome pathway (2, 6, 25). This model evolved from the observation that ubiquitination of purified nNOS by a purified ubiquitinating system is promoted by Hsp70 and inhibited by Hsp90 (25). We envision that as proteins undergo toxic or oxidative damage, ligand binding clefts open to expose hydrophobic residues as the initial step in unfolding. When Hsp90 can no longer cycle with the protein to inhibit ubiquitination, E3 ligases interacting with the substrate-bound Hsp70 target ubiquitin-charged E2 enzyme to the nascently unfolding substrate (6).

In Figs. 5, 7, and 8, we provide strong evidence that these two essential components of the chaperone machinery have opposing effects on nNOS ubiquitination in the cell, with Hsp70 promoting and Hsp90 inhibiting ubiquitination. The opposing effects of the two chaperones on substrates in the cell are required for a general model in which the Hsp90/Hsp70-based chaperone machinery makes the triage decision in protein quality control via the ubiquitin-proteasome pathway. In this respect, we note a report that treatment of cells with the irreversible thymidine kinase inhibitor CI-1033 induces ErbB-2 ubiquitination and proteasomal degradation (43). Like nNOS (26), CHIP serves as an E3 ligase for ErbB-2, and both CHIP and Hsp70 are co-immunoadsorbed with ErbB-2 from cells treated with the Hsp90 inhibitor geldanamycin (44). In the study by Citri et al. (43), it was noted that treatment with CI-1033 reduced ErbB-2 state of the heme/substrate binding cleft, reducing exposure of hydrophobic amino acids of the cleft interior that favor Hsp70 binding. Decreased binding of the Hsp70-dependent E3 ligase CHIP likely accounts for the decreased ubiquitination of nNOS seen with NNA (Fig. 2). Inasmuch as ubiquitination is the initial step leading to proteasomal degradation, this could account for the ability of NNA to stabilize nNOS (30).
interaction with Hsp90 and promoted its binding to Hsp70, consistent with the proposed role for the Hsp90/Hsp70-based chaperone machinery in protein quality control.

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