hsp90 Is Required for Heme Binding and Activation of Apo-Neuronal Nitric-oxide Synthase

GELDANAMYCIN-MEDIATED OXIDANT GENERATION IS UNRELATED TO ANY ACTION OF hsp90*

Received for publication, February 26, 2002
Published, JBC Papers in Press, March 28, 2002, DOI 10.1074/jbc.M201940200

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It is established that neuronal NO synthase (nNOS) is associated with the chaperone hsp90, although the functional role for this interaction has not been defined. We have discovered that inhibition of hsp90 by radicicol or geldanamycin nearly prevents the heme-mediated activation and assembly of heme-deficient apo-nNOS in insect cells. This effect is concentration-dependent with over 75% inhibition achieved at 20 μM radicicol. The ferrous carbonyl complex of nNOS is not formed when hsp90 is inhibited, indicating that functional heme insertion is prevented. We propose that the hsp90-based chaperone machinery facilitates functional heme entry into apo-nNOS by the opening of the hydrophobic heme-binding cleft in the protein. Previously, it has been reported that the hsp90 inhibitor geldanamycin uncouples endothelial NOS activity and increases endothelial NOS-dependent O2 production. Geldanamycin is an ansamycin benzoquinone, and we show here that it causes oxidant production from nNOS in insect cells as well as with the purified protein. At a concentration of 20 μM, geldanamycin causes a 3-fold increase in NADPH oxidation and hydrogen peroxide formation from purified nNOS, whereas the non-quinone hsp90 inhibitor radicicol had no effect. Thus, consistent with the known propensity of other quinones, geldanamycin directly redox cycles with nNOS by a process independent of any action on hsp90, cautioning against the use of geldanamycin as a specific inhibitor of hsp90 in redox-active systems.

The endothelial and neuronal isoforms of nitric-oxide synthase (NOS)† have been reported to exist in heterocomplexes with hsp90 (1, 2). These proteins join a list of numerous other signaling proteins, including steroid receptors, some transcription factors, and a variety of protein kinases, that are associated with and regulated by hsp90 (for a review, see Ref. 3). These signaling protein-hsp90 heterocomplexes are assembled in an ATP-dependent process by a five-protein system in which hsp90 and hsp70 are essential assembly components and Hop, hsp40, and p23 function as non-essential co-chaperones (4). One of the most studied hsp90-bound proteins is the glucocorticoid receptor (GR), which must be associated with hsp90 to have steroid binding activity (5, 6). Hsp90 binds directly to the ligand-binding domain of the GR (3), and biochemical data (7) coupled with data from GR mutants (8, 9) support the notion (6) that formation of a complex with hsp90 opens up a hydrophobic pocket in the ligand-binding domain to access by steroid. We have proposed a similar model for neuronal NOS (nNOS) in which the hsp90-based chaperone machinery acts in vivo to open the heme-binding cleft in heme-deficient apo-nNOS to access by heme (2).

In contrast to the observations with steroid receptors and nNOS, it has been proposed that hsp90 regulates endothelial NOS (eNOS) through an allosteric mechanism. García-Cardena et al. (1) were able to demonstrate direct activation of purified eNOS catalytic activity by purified hsp90 in the absence of ATP, hsp70, and the co-chaperones of the hsp90-based chaperone machinery. The proposed allosteric regulation of eNOS by hsp90 would be a unique mode of regulation in that other hsp90-regulated proteins require the ATP- and hsp70-dependent, multiprotein hsp90 chaperone system for their regulation. More recently, the same laboratory (10) has shown that geldanamycin, a specific inhibitor of hsp90, causes eNOS to produce superoxide in cells and tissues. From this, it was concluded that hsp90 is essential for eNOS-dependent NO production and that inhibition of hsp90 leads to uncoupling of eNOS and increased superoxide production due to conformational changes in hsp90 and eNOS. Other reports suggest that hsp90 facilitates the calmodulin-assisted dissociation of eNOS from caveolin, thereby opposing the inhibitory effect of caveolin on eNOS activity (11), and that eNOS-bound hsp90 recruits the protein kinase Akt, resulting in eNOS phosphorylation and sustained activation of the enzyme (12).

In the case of nNOS, it has been shown that geldanamycin causes an increased turnover of nNOS and a loss in nNOS activity, implying that hsp90 is important for the stability and function of the enzyme (2). Song et al. (13, 14) have shown that hsp90 in the absence of ATP, hsp70, or co-chaperones enhances the affinity of calmodulin binding to purified nNOS by ~10-fold. In our previous study (2), we were unable to show hsp90-mediated activation of the holo-nNOS, but we did show that geldanamycin treatment of Sf9 cells expressing nNOS inhibits heme-mediated activation of heme-deficient apo-nNOS. Thus, in analogy with the well studied steroid receptor model, we
on, the hsp90-based chaperone machinery plays a role in opening up the hydrophobic-binding cleft in apo-nNOS to facilitate the entry of heme into its binding site in the interior of the enzyme.

In this study, we show that the hsp90-inhibitors, geldanamycin and radicicol, cause a profound inhibition of heme-mediated activation of apo-nNOS activity in the insect opressor system. Inhibition of hsp90 is accompanied by a marked inhibition in heme binding and by an inability to form the dimeric state of nNOS, implying a role for hsp90 in heme-mediated assembly of the holo-enzyme. In the course of these studies, we have discovered that geldanamycin, an ansamycin benzoquinone, is capable of redox cycling with nNOS to produce reactive oxygen species in a manner similar to that characterized for other quinones (15). In contrast to geldanamycin, radicicol, a specific hsp90 inhibitor that is not a quinone, blocks heme binding by nNOS but does not cause the formation of reactive oxygen species. Thus, in contrast to the conclusion of Pritchard et al. (10) in their work with eNOS, hsp90 does not regulate oxidant generation from nNOS. This observation provides a caution against the use of geldanamycin as a mechanistic probe for hsp90 actions in redox-active systems in which drug-mediated oxidants may be generated.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). (6,7-3H)triamcinolone acetonide (35 Ci/mmol) and 125I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from PerkinElmer Life Sciences. (6R)-5,6,7,8-Tetrahydrobiopterin was purchased from Dr. Schirck (Laboratory (Jona, Switzerland). Protein A-Sepharose, iron protoporphyrin IX, l-arginine, myoglobin (horse heart), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, catalase, radicicol, NADPH, and NaD* were purchased from Sigma. The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from Transduction Laboratories (Lexington, KY). The cDNA for rat neuronal NOS was kindly provided by Dr. Solomon Snyder (The Johns Hopkins Medical School, Baltimore, MD). Hybridoma cultures producing the F1GIR monoclonal IgG against the GR were generously provided by Dr. Jack Bodwell (Dartmouth Medical School). The baculovirus for expression of the mouse GR was generously provided by Dr. Edwin Sanchez (Medical College of Ohio, Toledo, OH). Geldanamycin was obtained from the Drug Synthesis and Chemistry Branch, NCI National Institutes of Health (Bethesda, MD).

Methods

Expression of nNOS or GR in Sf9 Cells—Recombinant baculovirus containing nNOS cDNA was produced as described previously (21). Sf9 cells were grown in SFM 900 II serum-free medium (Invitrogen) supplemented with Cytopax (Kemp Biotechnology, Rockville, MD) in suspension cultures maintained at 27 °C with continuous shaking (150 rpm). Cultures (2 x 10^6 cells/ml) were infected in log phase of growth with recombinant baculovirus at a multiplicity of infection of 1.0. After 48 h, aliquots (3.0 ml) of the suspensions were treated with heme (24 μM), which was added as an albumin conjugate (16). In some samples, the cells were treated with geldanamycin, radicicol, or MeSO vehicle 20 min before the addition of heme. The final MeSO concentration was less than 0.4%. Cells were harvested, washed twice with 10 ml of ice-cold phosphate-buffered saline, and suspended in 350 μl of 10 mM Hepes, pH 7.5, containing 320 mM sucrose, 100 μM EDTA, 1.0 mM dithiothreitol, 10 μM γ-tremin inhibitor, 10 μM l-arginine, 2 μM aprotinin, and 6 mM phenylmethylsulfonyl fluoride. The cells were ruptured by Dounce homogenization, and the lysates were spun at 16,000 g for 10 min at 4 °C. The supernatant was collected, aliquoted, flash-frozen, and stored at −80 °C.

Sf9 cultures infected in log phase of growth with recombinant baculovirus containing the mouse GR cDNA at a multiplicity of infection of 3.0 were supplemented with 0.1% glucose at infection and 24 h post-infection as described by Sriniivasan et al. (17). Cells were harvested, washed in Hanks’ buffered saline solution, resuspended in 1.5 volumes of buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 20 mM molybdate, 1 mM phenylmethylsulfonyl fluoride) with 1 tablet of Complete-Mini protease inhibitor mixture per 3 ml of buffer, and ruptured by Dounce homogene-
ascites suspended in 200 μl of TEG buffer (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Prior to incubation with rabbit reticulocyte lysate, immunoadsorbed receptors were stripped of associated hsp90 by incubating the immunopellet for an additional 2 h at 4°C with 300 μl of 0.5 M NaCl in TEG. The pellets were then washed once with 1 ml of TEG followed by a second wash with 1 ml of Hepes buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity. Stripped GR immunopellets containing GR stripped of radicicol (H + GA), or in the presence of heme and 20 μM radicicol (H + GR, + RAD). Geldanamycin or radicicol was added 20 min prior to heme addition, and the abscissa represents time after the addition of heme. At the indicated times, cells were harvested, and nNOS activity was assayed by the oxyhemoglobin oxidation method.

RESULTS
Geldanamycin and Radicicol Inhibit Heme-mediated Activation of Apo-nNOS in Sf9 Cells—Sf9 cells expressing heme-deficient apo-nNOS were incubated in the presence of 24 μM heme (+Heme), in the presence of heme and 20 μM geldanamycin (+Heme, + GA), or in the presence of heme and 20 μM radicicol (+Heme, + RAD). Geldanamycin or radicicol was added 20 min prior to heme addition, and the abscissa represents time after the addition of heme. At the indicated times, cells were harvested, and nNOS activity was assayed by the oxyhemoglobin oxidation method.

Fig. 1. Both radicicol and geldanamycin inhibit heme-mediated apo-nNOS activation in Sf9 cells. Sf9 cells expressing heme-deficient apo-nNOS were incubated in the absence of heme (Untreated), in the presence of 24 μM heme (+Heme), in the presence of heme and 20 μM geldanamycin (+Heme, + GA), or in the presence of heme and 20 μM radicicol (+Heme, + RAD). Geldanamycin or radicicol was added 20 min prior to heme addition, and the abscissa represents time after the addition of heme. At the indicated times, cells were harvested, and nNOS activity was assayed by the oxyhemoglobin oxidation method.

Both geldanamycin (open squares) and radicicol (closed squares) with complete inhibition at 1 μM. Geldanamycin (open squares) is slightly less potent, producing nearly complete inhibition at 5 μM. The concentration dependence of this in vitro inhibition is similar to that reported for in vitro inhibition of hsp90 ATPase activity by geldanamycin and radicicol (26). The higher concentrations required for inhibition of nNOS activation in Sf9 cells are similar to those found in the recent report by Pritchard et al. (10), who used 18 μM geldanamycin to cause eNOS-dependent superoxide production in bovine coronary endothelial cells.

Both Geldanamycin and Radicicol Inhibit Heme-initiated Dimerization of Apo-nNOS in Sf9 Cells—The inactive, heme-deficient, monomeric apo-nNOS must form a heme-bound homodimer in order for the enzyme to produce nitric oxide (27). Under in vivo conditions, this assembly process gives rise to a very stable dimeric state of the enzyme that can be observed on low temperature SDS-PAGE gels as an SDS-resistant dimer (23). As shown in Fig. 3A, in Sf9 cells, the monomeric form of the enzyme is produced in the absence of exogenous heme (lane 1). After the addition of exogenous heme for 30 min, the nNOS exists, in large part, as an SDS-resistant dimer (lane 2). When the cells are treated with heme in the presence of 20 or 40 μM geldanamycin, the SDS-resistant dimer is substantially decreased (lanes 3 and 4), and radicicol decreases the amount of SDS-resistant dimer to a similar extent at the same concentrations (lanes 5 and 6).

The dimeric form of nNOS can also be measured under non-denaturing conditions in which detergent is omitted. Under these conditions, it is possible to see a dimeric form of nNOS that is not SDS-resistant (28). As shown in Fig. 3B, nNOS exists as a mixture of monomeric and dimeric forms (lane 1). After the addition of exogenous heme, the monomeric nNOS is converted to the dimeric form (lane 2). When the addition of heme is carried out in the presence of 20 μM geldanamycin (lane 3) or 20 μM radicicol (lane 4), the conversion of the monomer to the dimer is inhibited.

Radicicol Hinders Functional Heme Insertion into Apo-nNOS—The initial step in the heme-mediated dimerization of apo-nNOS in vitro is the insertion of heme into the protein and ligation of the heme iron to a cysteine (28). This heme complex has a characteristic absorbance in the 450-nm region in its ferrous carbonyl state. Although this complex can be readily detected in Sf9 cytosols, a heme-derived chromophore at 420
nm interferes with the quantification of the P450. To minimize the contribution of this 420-nm absorbing compound, we have partially purified the nNOS by the use of a 2 S’-ADP-Sepharose affinity column and then measured the P450 spectrum (Fig. 4, inset). As shown in Fig. 4, the amount of P450 was quantitated and compared with the cytosolic nNOS activity. The addition of heme (Heme) to Sf9 cells expressing apo-nNOS increased nNOS activity and P450 content 5-fold when compared with that found when heme was not added (Unt). When the cells were treated with heme in the presence of 20 or 40 μM radicicol (Heme + 20 μM RAD or Heme + 40 μM RAD), the nNOS activity and P450 content remained at nearly the level of the untreated cells (Unt). The addition of 40 μM radicicol after activation of apo-nNOS with heme did not cause a decrease in nNOS activity (data not shown). Thus, it appears that radicicol does not act to inhibit the active form of nNOS. As will be described in more detail below, geldanamycin caused the oxidative destruction of heme and therefore was not used in these studies.

Geldanamycin, but Not Radicicol, Causes Oxidant Production from nNOS in Sf9 Cells—To determine whether heme availability could be a mechanism for the reduction in dimeric nNOS observed after treatment with the hsp90 inhibitors, we measured the amount of heme in the Sf9 cells by reverse phase HPLC. Fig. 5A shows the HPLC profiles at 400 nm of cytosolic samples prepared from Sf9 cells. Treatment of Sf9 cells overexpressing nNOS with exogenous heme and subsequent analysis of the cell cytosol gave a major peak at 22 min corresponding to heme (upper panel). This represents an 7-fold increase in heme over that found in Sf9 cells that have not been treated with exogenous heme (data not shown). Treatment of cells with exogenous heme and 20 μM geldanamycin causes 80% decrease in the peak corresponding to heme (middle panel), whereas 20 μM radicicol does not affect the amount of heme (lower panel). The peak areas for heme were quantitated, as shown in Fig. 5B. The loss of heme is dependent on the concentration of geldanamycin (open squares), whereas radicicol had no substantial effect (closed squares). In that both compounds are hsp90 inhibitors, the heme loss appears unrelated to hsp90. The decrease in cytosolic heme may contribute to the inhibition of heme-mediated activation of Sf9 cell nNOS seen with geldanamycin treatment, but an effect on heme cannot explain any inhibition of nNOS activation by radicicol.

We further examined the heme levels in the medium to determine whether heme availability is a determinant of geldanamycin action. As shown in Fig. 6A on the 400-nm profile (solid lines), the heme in the medium of radicicol-treated cells (lower panel) was not different from that of untreated cells (upper panel). However, 20 μM geldanamycin (middle panel) causes a 70% decrease in heme as well as the formation of a new peak on the 400-nm profile (solid line) that co-elutes with albumin, which is seen on the 220-nm profile (dashed line). This new heme product likely represents an altered heme product that is irreversibly bound to albumin. Large amounts of albumin are present in the medium because it was added to stabilize the heme as a heme-protein conjugate. The formation of altered heme products occurs when hemoproteins, including heme-albumin conjugates (29), react with reactive intermediates, such as hydrogen peroxide (21, 30). As shown in Fig. 6B, this notion that hydrogen peroxide is involved in the formation of the altered heme product is supported by the finding that catalase prevents the heme loss (cf. GA with GA + CAT) as well as the formation of the altered heme adduct (data not shown). Hydrogen peroxide treatment of the medium containing heme causes the loss of heme ($H_2O_2$) and the formation of the altered heme adduct (data not shown). Thus, hydrogen peroxide is formed and causes the alteration of the heme in the medium. Moreover, hydrogen peroxide formation requires nNOS, as no heme loss was observed in non-infected cells treated with
NADPH oxidation and hydrogen peroxide formation (addition, the heme-deficient apo-nNOS was able to catalyze a variety of other quinones (15). Lead to redox cycling of geldanamycin. The flavin domain of the heme domain, mediates the electron transfer reactions that APO inhibitors that act on the heme active site of nNOS could prevent assembly of monomeric apo-nNOS to the enzymatically active homodimeric form. Thus, we conclude that the hsp90-based chaperone machinery plays a role in facilitating functional heme insertion.

This notion of the insertion of a hydrophobic heme into nNOS is consistent with the action of the hsp90-based chaperone machinery on the GR, where the hydrophobic ligand-binding cleft is opened to allow access by steroid (7–9). In a similar manner, the chaperone machinery may favor the opening of the heme-binding cleft and facilitate heme entry. The presence of hydrophobic clefs is a universal feature of all properly folded proteins, and the ability of the hsp90-based chaperone machinery to recognize the general topological regions where clefs merge with the surface of a protein is consistent with the notion that the cleft opening and facilitated binding of hydrophobic compounds are primary functions of the chaperone machinery. It is likely that the hsp90-based chaperone machinery facilitates binding of a number of hydrophobic ligands and prosthetic groups through stabilization of a partially unfolded state of native acceptor proteins.

The role of hsp90 in heme insertion is also consistent with the known association of hsp90 with nNOS in vivo and in vitro as well as the enhanced degradation of nNOS seen after inhibition of hsp90 in cells (2). nNOS is known to be proteolyzed by a ubiquitin-proteasomal system, and the monomeric apo-nNOS is posttranslationally ubiquitinated (31). Inhibition of hsp90 and decreased heme insertion into apo-nNOS would favor the monomeric form and thus enhance the proteolysis and turnover of nNOS in cells.

In the course of our study, we discovered that geldanamycin, unlike radicicol, mediates the formation of oxidants from nNOS that cause the oxidative alteration of heme. The oxidant ap-
pears to be hydrogen peroxide since catalase prevents against heme alteration, and similar heme products are formed after treatment with exogenous hydrogen peroxide. Treatment of endothelial cells with geldanamycin causes eNOS-dependent oxidant production, leading Pritchard et al. (10) to conclude that inhibition of ATP-dependent conformational changes in hsp90 uncouples eNOS activity to increase O$_2^·$ generation by the enzyme. In essence, it was concluded that the ADP-versus ATP-dependent conformation of eNOS-bound hsp90 determined O$_2^·$ versus NO production. In the case of nNOS, we have found that geldanamycin-mediated oxidant production is a direct action on nNOS independent of hsp90. This is based on the finding that geldanamycin redox cycles and forms oxidants with purified nNOS and that radicicol, which is an equally potent hsp90 inhibitor, does not cause oxidant production in cells or with purified nNOS. The redox cycling of geldanamycin, an ansamycin benzoquinone, is consistent with the known ability of nNOS to redox cycle other quinones via the flavin redox center (15). Thus, the redox cycling of geldanamycin precludes the use of this inhibitor in the study of hsp90 effects in redox-active systems.

Acknowledgments—We thank Solomon Snyder, Lance Pohl, Jack Bodwell, and Edwin Sanchez for providing the cDNAs and antibodies used in this work.

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