

Estrogen Stimulates Heat Shock Protein 90 Binding to Endothelial Nitric Oxide Synthase in Human Vascular Endothelial Cells

EFFECTS ON CALCIUM SENSITIVITY AND NO RELEASE*

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Kerry Strong Russell‡, M. Page Haynes‡, Teresa Caulin-Glaser‡, James Rosneck‡, William C. Sessa‡§, and Jeffrey R. Bender‡¶

From the ‡Division of Cardiovascular Medicine and Molecular Cardiobiology, Boyer Center for Molecular Medicine and §Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06536-0812

Estradiol (E₂) causes endothelium-dependent vasodilation, mediated, in part, by enhanced nitric oxide (NO) release. We have previously shown that E₂-induced activation of endothelial nitric oxide synthase (eNOS) reduces its calcium dependence. This pathway of eNOS activation is unique to a limited number of stimuli, including shear stress, the response to which is herbimycin-inhibitable. Consistent with this, herbimycin and geldanamycin pretreatment of human umbilical vein endothelial cells (HUVEC) abrogated E₂-stimulated NO release and cGMP production, respectively. These benzoquinone ansamycins are potent inhibitors of Hsp90 function, which has recently been shown to play a role in stimulus-dependent eNOS activation. As in response to shear, E₂ induced an Hsp90-eNOS association, peaking at 30 min and completely inhibited by the conventional estrogen receptor antagonist ICI 182,780. These findings suggest that Hsp90 plays an important role in the rapid, estrogen receptor-mediated modulation of eNOS activation by estrogen.

The cardioprotective effects of estrogen are well known and appear to be mediated by both effects on lipoprotein/lipid profiles and other as yet incompletely defined direct vascular effects. Estrogen induces both rapid and delayed effects on the cardiovascular system. A number of clinical studies have shown that estrogen, administered in physiologic concentrations, causes endothelium-dependent vasodilation (for a review, see Guetta and Cannon (1)). This is likely to be mediated, in part, by augmentation of nitric oxide (NO)¹ release because estrogen has been demonstrated to produce a significant increase in the levels of bioavailable NO (2–5).

Using an *in vitro* model system of vascular endothelial cells (EC), we and others have demonstrated that EC secrete NO rapidly after stimulation with estrogen (6–8). Our data have

also shown that the increase in NO release is due to activation of the endothelial form of NO synthase (eNOS or NOS 3). This activation appears to significantly reduce the calcium-calmodulin dependence of the enzyme, and we have further demonstrated that treatment of human umbilical vein EC (HUVEC) with estrogen, under conditions which result in NO release, does not result in a measurable increase in free intracellular calcium (6). This pathway of eNOS activation is unique to a limited number of stimuli, which include the sustained phase of shear stress-induced vasodilation (9–11).

The activity of eNOS has been shown to be regulated by multiple factors including: post-translational fatty acid modification (including palmitoylation and myristoylation (12–15)); phosphorylation (16, 17); alterations in subcellular localization, particularly partitioning into plasmalemmal caveolae (13, 18); and binding to other proteins or cofactors, including tetrahydrobiopterin (BH₄) (19), calcium-dependent calmodulin (20), caveolin-1 (21), eNOS itself (22), and most recently, heat shock protein 90 (Hsp90) (23).

Binding of eNOS to Hsp90 has been shown to occur in response to vascular endothelial growth factor (VEGF), histamine, and shear stress; and eNOS activity is increased in a dose-dependent manner in the presence of Hsp90 (23). Furthermore, geldanamycin also significantly decreases activation of eNOS by VEGF and histamine as well as acetylcholine-induced endothelium-dependent relaxation (23). The benzoquinone ansamycin antibiotics, which include herbimycin and geldanamycin, have been shown to disrupt a number of tyrosine kinase signaling pathways and reverse cellular transformation by Src (24), leading to their use as “specific” tyrosine kinase inhibitors. However, more recent evidence suggests that most of these effects may be mediated by their ability to impair Hsp90 function rather than a direct effect on the protein kinase itself (25).

The mechanism by which estrogen regulates eNOS activity is the focus of this and ongoing studies in our laboratory. In this report, we demonstrate that the ansamycins inhibit estrogen-mediated HUVEC NO release, cGMP production, and calcium-independent NOS activity. We show that 17 β -estradiol (E₂) promotes a rapid Hsp90-eNOS association, an effect that is blocked by a conventional estrogen receptor (ER) antagonist. These findings demonstrate that Hsp90 plays an important role in the regulation of eNOS function by estrogen.

EXPERIMENTAL PROCEDURES

Materials—17 β -Estradiol and water-soluble 17 β -estradiol (estradiol in cyclodextrin) were purchased from Sigma. Estradiol stocks were prepared in ethanol and stored at –20 °C. The final concentration of ethanol in all cell cultures was \leq 0.01%. All controls were treated with identical concentrations of ethanol (vehicle) simultaneously with the addition of estradiol. Water-soluble estradiol stocks were made fresh for each experiment in sterile phosphate-buffered saline. ICI 182,780 was

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¶ To whom correspondence should be addressed: Boyer Center for Molecular Medicine, 454C, Yale University School of Medicine, 295 Congress Ave., New Haven, CT 06536-0812. Tel.: 203-737-2223; Fax: 203-737-2293; E-mail: jeffrey.bender@yale.edu.

¹ The abbreviations used are: NO, nitric oxide; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein EC; BH₄, tetrahydrobiopterin; Hsp90, heat shock protein 90; VEGF, vascular endothelial growth factor; NOS, nitric-oxide synthase; E₂, 17 β -estradiol; ER, estrogen receptor; NOx, nitric oxide; PAGE, polyacrylamide gel electrophoresis; iNOS, inducible form of NOS; L-NAME, N^G-nitro-L-arginine methyl ester; GA, geldanamycin.

purchased from Zeneca Pharmaceuticals. Herbimycin was purchased from Life Technologies, Inc., and geldanamycin was from Calbiochem. Herbimycin and geldanamycin were dissolved in Me₂SO and stored at -20 °C. The final concentration of Me₂SO in all cultures and controls (in all experiments where herbimycin was used) was ≤0.1%. Antibodies were purchased from the following sources: anti-eNOS (N30020), Transduction Laboratories; anti-Hsp90 (SPA-845), StressGen; and anti-calmodulin, Upstate Biotechnology. All other reagents were purchased from Sigma unless otherwise indicated.

Cell Isolations and Culture—HUVEC were isolated from single female donors as described previously (26). Cells were routinely passaged on gelatin-coated plates in M199 with 15% fetal bovine serum, bovine endothelial cell growth supplement (50 μg/ml, Collaborative Research), and heparin (100 μg/ml). Prior to E₂ stimulation, cells were cultured in phenol red-free M199 or Dulbecco's modified Eagle's medium with gelding serum (Sigma, lot 114H4629, <1.0 pg/ml estradiol) and no endothelial cell growth supplement/heparin for 24–48 h.

NOx Analysis—NOx (including NO₂, NO₃, and nitrosothiols) was measured in aqueous media after a 1-h equilibration time using a chemiluminescent assay and a commercial NO analyzer (Siemens) as described previously (14). Subsequently, cell monolayers were trypsinized, and single-cell suspensions were counted to determine the number of cells present in each experimental sample. NOx levels are expressed in terms of total NOx liberated (picomoles/total cell number).

NOS Activity Assays—eNOS activity was assayed in total cell lysates as described previously (6). Briefly, cell lysates were made in the presence of EDTA (0.1 mM) and EGTA (0.1 mM) to chelate free calcium released with cell disruption. To determine the maximal NOS activity in each sample, exogenous calcium (2.5 mM), calmodulin (100 nM), BH₄ (30 μM), and NADPH (1 mM) were added. The amount of NOS activity in each sample determined in the presence of all these cofactors was set at 100%, and NOS activity in each sample under other conditions (e.g. without added calcium and calmodulin) was calculated as a percentage of this activity. Subsequently, conversion of L-[³H]arginine to [³H]citrulline was measured by column chromatographic separation of these two molecules and liquid scintillation counting.

Coimmunoprecipitations of eNOS, Calmodulin, and Hsp90—HUVEC monolayers were stimulated as described in the figure legends. Total cell lysates were made on ice in buffer containing: 20 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, and 20 mM NaMoO₄. Immunoprecipitations were carried out in the presence of primary antibody (anti-eNOS N30020, 1–2 μg/mg of total cell protein) for 2 h at 4 °C, followed by overnight incubation with a 1:1 protein A:protein G-Sepharose slurry. Following washes, immunoprecipitates were boiled in sample buffer, separated by SDS-PAGE, transferred, and immunoblotted. The primary antibodies used for immunoblotting were anti-eNOS (1:2500), anti-calmodulin (1:1000), or anti-Hsp90 (1:500). Blots were then probed with secondary antibodies coupled to horseradish peroxidase and visualized by autoradiography using a chemiluminescent substrate (ECL, Amersham Pharmacia Biotech).

cGMP Assays—cGMP was acid-extracted from intact cell monolayers as described previously (27). Samples were pH-neutralized, and cGMP levels were measured using a radioimmunoassay kit (Biomedical Technologies Inc.).

RESULTS

Estrogen Does Not Alter the Binding of Calmodulin to eNOS—Our previous data demonstrate that E₂ stimulation of HUVEC alters the calcium dependence of the enzyme toward a more calcium-independent state (6). The inducible form of NOS (iNOS) also shows a relative state of calcium independence. Active enzyme complexes of iNOS have been demonstrated to contain calmodulin, even in the presence of only trace levels of calcium (28). Calmodulin binding appears to impart a crucial conformational change in the complex, which allows NOS activation without an increase in free intracellular calcium. Using this as a model, we asked whether E₂ stimulation could induce increased binding of calmodulin to eNOS under conditions in which E₂ does not appear to increase free intracellular calcium levels (6). As shown in Fig. 1, E₂ does not affect the total amount of calmodulin bound to eNOS in HUVEC. This suggests that the mechanism by which E₂ activates eNOS does not involve significant changes in calmodulin binding to the enzyme complex in the absence of increased cytosolic calcium.

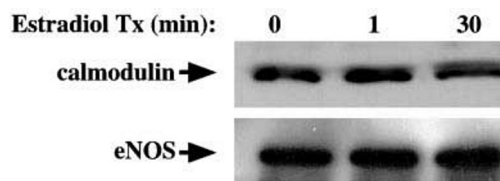


FIG. 1. Effect of 17β-estradiol on calmodulin/eNOS association. Confluent HUVEC monolayers were incubated with E₂ (50 ng/ml) for the indicated times at 37 °C. Immunoprecipitations of eNOS were carried out as described under “Experimental Procedures.” Blots were probed first with an anti-calmodulin antibody (top panel) and reprobed with an anti-eNOS antibody (bottom panel). Results are representative of three separate experiments. In the experiment shown, a 3-fold increase in NO was detected in E₂-treated cells (30-min time point).

Herbimycin Blocks Estrogen-induced NO Release and Calcium Independence of eNOS—The rapid augmentation in NO release induced by E₂ suggests a post-translational event. Because calcium-independent, flow-mediated vasodilation is abrogated by herbimycin (10, 11), experiments were performed to evaluate whether the effect of E₂ on eNOS is also herbimycin-inhibitable. As shown in Fig. 2, pretreatment of HUVEC with herbimycin completely abolishes the increase in NO production seen after E₂ treatment. Furthermore, this herbimycin effect is also seen at the level of eNOS activity. That is, in cell-free assays, eNOS activity is undetectable in calcium/calmodulin-free conditions, as expected (Fig. 3). As we have previously shown, eNOS recovered from HUVEC pretreated for 24 h with E₂ exhibits maximal activity in the same calcium/calmodulin-free conditions. However, herbimycin pretreatment largely abrogates the calcium/calmodulin independence of this enzyme induced by E₂, an effect we have also previously shown to be inhibited by conventional ER antagonists (6). Basal and E₂-stimulated activity were inhibited by L-NAME, demonstrating that the activity measured was due specifically to NOS activation (Fig. 3 and data not shown).

Estrogen Increases Hsp90-eNOS Association—As described above, herbimycin is a member of the ansamycin benzoquinone antibiotics whose cellular effects have previously been described as selective inhibitors of tyrosine kinase receptor activity. More detailed biochemical analysis of these molecules has revealed that they have significant effects on the function of the molecular chaperone Hsp90. Because herbimycin has a potent effect on E₂-stimulated NO release and Hsp90 appears to have a regulatory effect on eNOS function (23), we evaluated whether E₂ stimulation of human EC promotes an Hsp90-eNOS association, one that could be critical in modulating eNOS activity. Figs. 4 and 5 demonstrate that E₂ rapidly stimulates Hsp90 association with eNOS. In contrast to the VEGF response, in which maximal Hsp90-eNOS association can be seen within 1 min, the E₂ response peaks at 30 min, at which time maximal association occurs (Fig. 4). This pattern is similar to that seen in shear stress-activated EC (23).

E₂-stimulated Hsp90-eNOS association is completely abrogated by the ER antagonist ICI 182,780 (Fig. 5, compare lanes 4 and 5 with lane 2). The finding that this antagonist blocks both E₂-stimulated NO release and the induced molecular complex formation is consistent with Hsp90-eNOS association playing a critical role in the pathway by which E₂ activates eNOS and modulates NO release.

Geldanamycin Decreases HUVEC cGMP Production Induced by Estrogen—To test whether the effects of herbimycin on E₂-stimulated NO release are also seen with other ansamycin benzoquinones, HUVEC intracellular cGMP levels (reflective of biofunctional NO production) were measured in E₂-stimulated cells pretreated with geldanamycin (GA). As opposed to herbimycin, GA does not require prolonged pretreatment of the cells,

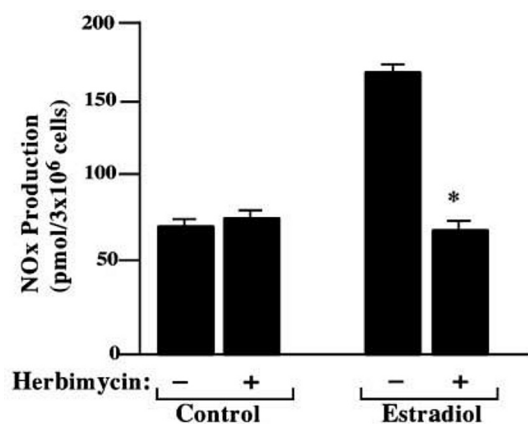


FIG. 2. Effect of herbimycin on estrogen-induced NO release. Confluent HUVEC monolayers were incubated with herbimycin (1 μ g/ml) or vehicle overnight (12 h) at 37 $^{\circ}$ C. Monolayers were then stimulated with E_2 (10 ng/ml) or vehicle for 30 min at 37 $^{\circ}$ C, followed by a phosphate-buffered saline medium change for 1 h and measurement of NOx by chemiluminescence. Data represent the mean \pm S.D. of three experiments. *, $p < 0.05$ compared with NOx production in the estradiol-treated cells without herbimycin.

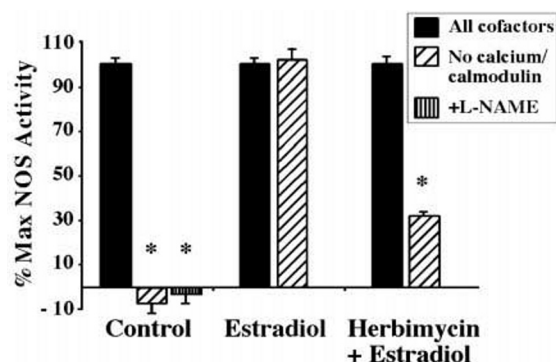


FIG. 3. Effect of 17 β -estradiol on calcium-dependent eNOS activity. Confluent HUVEC monolayers were incubated with herbimycin (1 μ g/ml) or vehicle overnight (12 h) at 37 $^{\circ}$ C. Monolayers were then stimulated with E_2 (50 ng/ml) or vehicle for 60 min at 37 $^{\circ}$ C. eNOS activity was assayed using an *in vitro* L-[³H]arginine to L-[³H]citrulline conversion enzymatic assay, as described under "Experimental Procedures." To determine relative calcium/calmodulin dependence of eNOS, lysates were made in the presence of calcium chelators. *All cofactors*, eNOS activity in the presence of added BH_4 , NADPH, calmodulin, and calcium; *No calcium/calmodulin*, eNOS activity in the presence of added BH_4 and NADPH but no added calcium or calmodulin; *+L-NAME*, eNOS activity in the presence of 10 mM L-NAME (a competitive inhibitor of eNOS) added at the same time as all other cofactors. The "all cofactors" sample within each experimental group is standardized to 100% NOS activity, and the altered condition samples within each group are compared on a relative (0–100%) scale. Data represent the mean \pm S.D. of three experiments. *, $p < 0.05$ compared with treatment-matched cells in the presence of all cofactors.

thereby allowing evaluation of ansamycin effects over a much shorter time span (20 min). As we have previously shown, the rapid E_2 -stimulated increase in cGMP production is inhibited by the ER antagonist ICI 182,780. GA completely abrogates this E_2 -stimulated increase in cGMP production (Fig. 6). These results support the concept that ansamycins, as a class, prevent E_2 -mediated modulation of eNOS activity by disrupting critical Hsp90-dependent signaling events.

DISCUSSION

Both estrogen and NO play critical roles in blood vessel development, function, and remodeling. Understanding the interplay between these two powerful, interdependent vascular modulators may be a first step in designing strategies for treating conditions such as ischemic, diabetic, and postmeno-

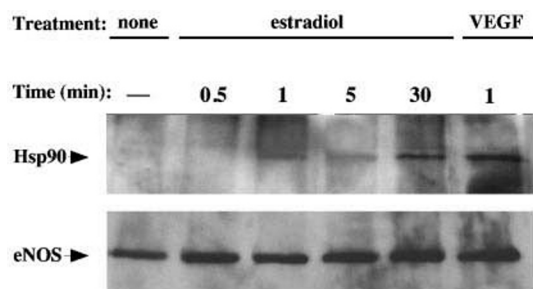


FIG. 4. Effect of 17 β -estradiol on Hsp90-eNOS association. Confluent HUVEC monolayers were incubated with E_2 (50 ng/ml) or VEGF (50 ng/ml) for the indicated times at 37 $^{\circ}$ C, after which eNOS was immunoprecipitated. After SDS-PAGE and transfer, the immunoprecipitates were probed first with an anti-Hsp90 antibody (*top panel*) and reprobbed with an anti-eNOS antibody (*bottom panel*). Results are representative of three separate experiments.

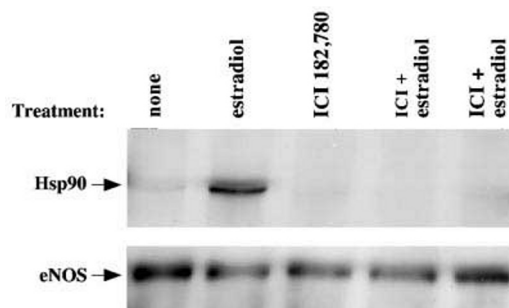


FIG. 5. Effect of an ER antagonist on 17 β -estradiol-induced Hsp90-eNOS association. Confluent HUVEC monolayers were incubated with E_2 (10 ng/ml) and/or ICI 182,780 (10 μ M) for 30 min at 37 $^{\circ}$ C, after which eNOS was immunoprecipitated. After SDS-PAGE and transfer, the immunoprecipitates were probed first with an anti-Hsp90 antibody (*top panel*) and reprobbed with an anti-eNOS antibody (*bottom panel*). Results are representative of three separate experiments.

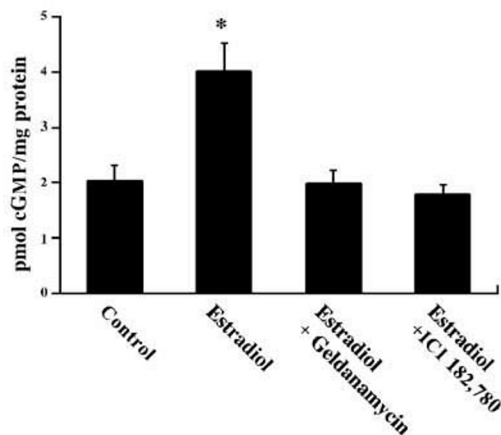


FIG. 6. Effect of geldanamycin on estrogen-induced cyclic GMP production. Confluent HUVEC monolayers were incubated with geldanamycin (5 μ g/ml) or vehicle for 20 min at 37 $^{\circ}$ C. Monolayers were then stimulated with E_2 (10 ng/ml) in the presence or absence of geldanamycin or ICI 182,780 (10 μ M) for 20 min at 37 $^{\circ}$ C. Monolayers were then acid-extracted, and cGMP levels were determined by radioimmunoassay. Data represent the mean \pm S.D. from a representative experiment. *, $p < 0.05$ compared with control, estradiol + geldanamycin, and estradiol + ICI 182,780.

pausal vascular dysfunction. Our data provide answers to some important initial questions regarding regulation of eNOS activity by estrogen.

Investigations into the similarities and differences between the three known isoforms of NOS have led to insights into how cellular secretion of NO can be a critical step in processes as

diverse as immune responses and neuronal signaling. The finding that estrogen-activated eNOS displays a decrease in calcium dependence led us to hypothesize that estrogen causes eNOS to function similarly to its "calcium-independent" counterpart, iNOS, by allowing a shift in the ability of eNOS to interact with calmodulin under conditions of very low free intracellular calcium. This hypothesis was supported by our previous data showing that addition of histamine to estradiol-stimulated HUVEC does not result in a further increase in NOS activity over that seen with estradiol alone (6). However, our current data demonstrate that easily detectable levels of calmodulin are eNOS-bound in unstimulated HUVEC and that E_2 does not increase these levels. The conformation of calmodulin, when bound to its target proteins, determines its ability to promote enzyme activation (29). It is possible that estrogen alters the conformation and/or function of calmodulin already associated with eNOS, thereby modulating its interaction with the recently described eNOS autoinhibitory domain (30).

There are several similarities in the pathway by which estrogen and shear stress augment eNOS activity. Both stimuli elicit a relative calcium independence of the enzyme (9). Both shear and estrogen effects are inhibited by "protein tyrosine kinase inhibitors." Either genistein or herbimycin has been used in a majority of these studies. Although the effect of genistein on E_2 responses can be difficult to interpret, based on its phytoestrogenic properties, it has been used extensively as a protein tyrosine kinase inhibitor. Genistein has been shown to reduce shear stress-induced NO release, with a proposed modulatory effect on shear-induced eNOS phosphorylation (11). Isometric contraction of rabbit aortic rings is another dynamic model of induced NO release in which eNOS becomes relatively calcium/calmodulin-independent. However, eNOS activation by isometric contraction is unaffected by genistein (31). In contrast, herbimycin effectively inhibits isometric contraction-mediated NO release. In those studies, attempts were made to correlate modulation of NO release with alteration in patterns of protein tyrosine phosphorylation. Several proteins, but not eNOS itself, were shown to be tyrosine-phosphorylated in response to isometric contraction. As with NO analyses, herbimycin, but not genistein, dramatically decreased aortic EC phosphotyrosine content in response to isometric contraction. These findings support the concept that herbimycin effects on stimulated eNOS may occur through mechanisms other than direct inhibition of eNOS phosphorylation. Indeed, our data indicate that ansamycin effects on E_2 -stimulated eNOS activation involve their ability to disrupt Hsp90 function. However, we cannot exclude the possibility that some of these effects are due to alteration in tyrosine phosphorylation of proteins, which may be involved in eNOS regulation.

The ansamycins specifically bind the amino-terminal portion of Hsp90 in a region that is required for ATP binding and consequently decrease this interaction (32, 33). It has recently been shown that the phosphorylation state of the adenine nucleotide that is bound to Hsp90 appears to affect the formation of Hsp90-associated multimolecular complexes. In that study, GA inhibited Hsp90 complex formation with p23 and immunophilins (34). Interference with Hsp90-pp60^{v-src} complex formation by herbimycin or GA has also been shown to block v-src-mediated cellular transformation (24). In other systems, the effects of ansamycins on Hsp90 function appear to occur in the absence of physical disruption of complex formation between the regulated protein and Hsp90. 1) GA interferes with steroid hormone receptor function without disrupting hormone receptor/Hsp90 heterocomplex formation (35); and 2) interference with Ah receptor function by GA occurs in the absence of disruption of its physical association with Hsp90 (36). We also

did not observe a quantitative decrease in E_2 -induced Hsp90-eNOS complex formation in the presence of herbimycin (data not shown).

In the cytosol, classical ligand-free ERs ($ER\alpha$) are part of a multimolecular complex that includes several chaperones, including Hsp90. It is unlikely that the key molecular switch mediating E_2 /ER activation of eNOS is induction of a shift in the specific Hsp90 pool from ER- to eNOS-bound, because the heat shock proteins are among the most abundant cytoplasmic proteins. Therefore, the fraction of Hsp90 bound to ER even under E_2 -deprived conditions is unlikely to be a limiting factor in the amount of Hsp90 available for binding to other proteins such as eNOS. Furthermore, estrogen receptor antagonists can also cause dissociation of Hsp90 from ER (37), which would predict that these antagonists could cause increased Hsp90-eNOS binding and eNOS activation (in contrast to our findings). Although both ansamycins and ER antagonists can disrupt E_2 /ER signaling, our data suggest that in the case of eNOS activation by E_2 , these agents are not acting identically. ICI 162,780 blocks E_2 -induced Hsp90-eNOS association whereas GA does not. This is particularly interesting in light of other data from our laboratory suggesting that eNOS activation may be mediated by a surface receptor for estrogen,² and this "surface ER" may have differential requirements for Hsp90 function as compared with traditional "nuclear" ERs.

The pathway through which E_2 activates rapid signaling events is currently poorly defined. Non-genomic effects of E_2 /ER have been described in multiple systems and include rapid alterations in phosphorylation of intracellular proteins, levels of intracellular cAMP, and activation of the p21^{ras}/mitogen-activated protein kinase pathway (38–40). Estrogen has also been shown to activate the phosphatidylinositol 3-OH kinase/Akt signaling pathway in breast and uterine cells (41, 42). Akt activation has been shown to occur in a calcium-independent manner after stimulation by growth factors (*e.g.* epidermal growth factor (43)) and by shear stress (44). Recently, we and others have shown that eNOS is serine-phosphorylated in response to Akt activation (44, 45). This modification leads to activation of eNOS in a manner that appears to reduce its calcium dependence. Interestingly, Akt-mediated eNOS activation can be seen in response to both calcium-dependent (*e.g.* VEGF) and calcium-independent (*e.g.* shear stress) stimuli. The finding that herbimycin A does not block shear stress-induced Akt activation (46) suggests either that functional Hsp90-eNOS association is a critical downstream mediator of eNOS activation by Akt or that the pathways by which Akt and Hsp90 regulate eNOS activity are independent. The idea that these may represent independent pathways is also supported by the finding that the phosphatidylinositol 3-OH kinase inhibitors wortmannin and LY294002 only partially block VEGF- or insulin-stimulated NO release (47, 48), whereas wortmannin completely abrogates eNOS serine phosphorylation (45). Furthermore, recent studies have defined an autoinhibitory loop within the eNOS reductase domain (30). Deletion of this loop greatly reduces the calcium requirements of the enzyme. It is possible that estrogen induces a bipartite eNOS activation response, one that depends on Akt and affects the conformation of the autoinhibitory domain and the other that recruits required effectors through an induced Hsp90-eNOS association. Experiments are currently under way to determine the interplay between estrogen-induced Hsp90-eNOS association and modulation of eNOS by Akt.

In summary, we have found that the mechanism by which estrogen activates eNOS involves an alteration in the binding

² K. Russell, P. Haynes, and J. Bender, submitted for publication.

of this enzyme to Hsp90. We hope that further elucidation of the individual steps in this pathway will allow development of novel therapeutic strategies for regulating endothelial NO production.

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