A Heat Shock Protein 90 Binding Domain in Endothelial Nitric-oxide Synthase Influences Enzyme Function*†

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Previous reports suggest heat shock protein 90 (hsp90) associates with endothelial nitric-oxide synthase (eNOS) to increase nitric oxide (NO) generation. Ansamycin inhibition of chaperone-dependent activity increases eNOS generation of superoxide anion (O2⁻) upon enzyme activation. In the present study we identify where hsp90 binds to eNOS using overlapping decoy peptides based on the amino acid (aa) sequence of eNOS (291–420). B1, B2, and B3 peptides inhibited hsp90 association with eNOS in cell lysates from proliferating bovine aortic endothelial cells. B2 (aa 301–320), common to both B1 and B3, decreased stimulated NO production and hsp90 association in bovine aortic endothelial cells. The B2/B3 peptide was redesigned to TSB2 that includes a TAT protein transduction domain and shortened to 14 aa. TSB2 impaired vasodilation of isolated facialis arteries in vitro and in vivo and increased eNOS-dependent O2⁻ generation in native endothelial cells on mouse aortas, whereas a control peptide, TSB(Ctr), which has the four glutamic acids in TSB2 substituted with alanine, showed no such effects. Site-directed mutagenesis of eNOS at 310, 314, 318, and 323 Glu to Ala yields an eNOS mutant that exhibited reduced hsp90 association and generated O2⁻ rather than NO upon activation. Together, these data demonstrate that hsp90 associates with eNOS at aa 310–323. Moreover, a decoy peptide based on this sequence is sufficient to displace hsp90 from eNOS and uncouple eNOS activity from NO generation. Thus, Glu310, Glu314, Glu318, and Glu323 in eNOS, although each does not do much by itself, synergistically they increase “cooperativity” in the association step that is critical for maintaining hsp90-eNOS interactions and promoting coupled eNOS activity. Such chaperone-dependent signaling may play an important role in modulating the balance of NO and O2⁻ generation from eNOS and, therefore, vascular function.

Previous studies showed that the association of heat shock protein 90 (hsp90) with eNOS played an important role in the generation of nitric oxide (NO) (1). Studies from this laboratory revealed that inhibition of hsp90 ATPase-dependent chaperone activity not only decreased stimulated NO generation but also increased eNOS-dependent O2⁻ production (2–4). These reports indicated that inhibiting hsp90-dependent signaling with eNOS allows eNOS to generate O2⁻ upon stimulation rather than NO. Because NO plays a central role in vascular biology, such changes in enzyme function will likely have a major impact on endothelial cell (EC) and vascular physiology.

An earlier report by Sessa and co-workers (5) showed, using a yeast two-hybrid system, that hsp90 interacted with eNOS at amino acid (aa) 300–400 of eNOS. Previous studies by Pagano and co-workers (6) showed that small peptides corresponding to a portion of gp91phox could act as decoy peptides to inhibit assembly of vascular NADPH oxidoreductase and, therefore, vascular O2⁻ generation. On the basis of these studies we reasoned that small peptides derived from the primary aa sequence of eNOS might be useful to determine where hsp90 bound on eNOS. Such knowledge might help in developing new tools for dissecting the cellular mechanisms by which hsp90 modulates eNOS function and subsequently EC-dependent vasodilation.

The objectives here were to determine where hsp90 binds to eNOS by developing decoy peptides that could disrupt hsp90 interactions with eNOS and determine the critical sites in eNOS that are responsible for hsp90-eNOS interactions and, thus, coupled eNOS activity. Here we identify the location where hsp90 binds to eNOS and show that decoy peptides derived from this site in eNOS are potent inhibitors of stimulated NO production and eNOS-dependent vasodilation.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Twelve overlapping peptides (B1–B12, 20-mers) were designed to span the entire region where hsp90 was reported to associate with eNOS (aa 291–420, bovine) (5). TAT protein transduction domain (PTD) (6), PEP1 (7), and

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3 The abbreviations used are: hsp, heat shock protein; eNOS, endothelial nitric-oxide synthase; EC, endothelial cell(s); BAEc, bovine aortic EC(s); aa, amino acids; HPLC, high performance liquid chromatography; MOPS, 4-morpholino-2-propanesulfonic acid; Ach, acetylcholine; PBS, phosphate-buffered saline; DHE, dihydroethidium; PTD, protein transduction domain; 2-OH-E;, 2-hydroxyethidium; I-NAMe, L-nitroarginine methyl ester; HEK cells, human embryonic kidney cells; WT, wild type; CaM, calmodulin; DETA-NONOate, diethylenetriamine NONOate.
An eNOS-derived Decoy Peptide Inhibits Vasodilation

eNOS-derived peptides were synthesized using Fmoc (N-(9-fluorenlylmethoxycarbonyl) chemistry in the Protein, Nucleic Acid Core Laboratory of the Medical College of Wisconsin. All peptides were made with C-terminal amide and N-terminal acetylation and HPLC-purified, and predicted molecular weights were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Preparation of Proliferating EC Lysates and Disruption of hsp90 Interactions with eNOS—BAEC were expanded and maintained in RPMI 1640 media containing 10% fetal bovine serum, antibiotics, and mycotics. BAEC cultures were passaged with trypsin-EDTA and used for experiments between passage 5 and 7 (4). Previous studies from this laboratory revealed that proliferating EC had much higher levels of hsp90 association with eNOS than confluent, non-proliferating EC (4). To identify which eNOS-derived peptide disrupts hsp90 interactions with eNOS, the 12 different B peptides were incubated with cell lysates from proliferating BAEC cultures. Proliferating BAEC in 100-mm dishes (10–20 dishes) were prepared as previously described (4) were lysed in modified radioimmune precipitation assay buffer (50 mM Tris HCl, pH 7.5, 1% Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 0.1% deoxycholic acid, 1× protease inhibitors (Sigma), 1× phosphatase inhibitors (Sigma)). Cell lysates were transferred to 1.5-ml microcentrifuge tubes, placed on ice, and sonicated 2 times (30 s), and cell debris was isolated by centrifugation (14,000g/100 g, 10 min, 4 °C) (4). Supernatants were removed from the cell debris and pooled, and cell protein was determined in the supernatants using BCA reagent (Pierce). An aliquot of the proliferating EC lysates (300 μg in 0.5 ml) was preclarified with protein A/G (20 μl of a 50% slurry, 2 h at 4 °C) and then incubated with each of the individual decoy peptides (final concentration 4.3 μM) for 2 h at 4 °C. Next, eNOS was immunoprecipitated from the incubations using H32 antibody from BioMol (1 μg/100 μg of cell lysate) as previously described (4). Protein A/G (50 μl of a 50% slurry) was added to isolate the immunoprecipitates. eNOS and its associated proteins were separated by SDS-PAGE (7.5% gel), transferred to nitrocellulose, and immunoblotted for eNOS and hsp90 as described (4).

Effects of eNOS-derived Peptides on Stimulated NO Production and hsp90 Association with eNOS in BAEC Cultures—BAEC were cultured and maintained in 100-mm culture dishes until confluent. The eNOS-derived peptide B2, which possesses aa sequences that are common to B1 and B3, was incubated for 30 min with PEP1, a protein transduction domain peptide (7) (mole:mole = 1:20) and then added to the BAEC cultures at a final peptide concentration of 5 nM. BAEC cultures were incubated overnight with either nothing (control), PEP1 alone (transduction control), or B2+PEP1. These pretreated BAEC cultures were washed and incubated in Hanks’ balanced salt solution containing L-arginine (25 μM) at 37 °C for 15 min to obtain basal NO production. The cultures were then stimulated with either 10 μM 5,6-dimethoxy-2-hydroxybenzimidazol-2′-carboxylic acid ethyl ester (DM-200), 50 μM L-NAME (200 μM) for 10 min followed by washout of excess, unreacted DHE with MOPS buffer alone. DHE is a cell-permeable dye that upon reaction with O2− is converted to a fluorescent 2-hydroxyethidium (2-OH-E+) product that can be quantified by fluorescent microscopy or HPLC (2, 10, 11). Aortas were quickly excised and examined for nuclear 2-OH-E+ staining (an index of O2− generation) using confocal fluorescent microscopy as previously described (2, 10). The intensity of the staining in each image taken from the confocal microscope was quantified by NIH image.

Vasodilation Studies—Previous studies showed that vasodilation of facialis arteries of mice was an eNOS-dependent mechanism (3, 4, 9). To determine the effects of B2 on vasodilation, we redesigned B2 to contain a PTD. The B2 peptide was also shortened to 14 aa (ELVLEVPLEHPTLE) with an Ala added at the N terminus with the TAT PTD (RKKRRQRRR-A-ELVLEVPLEHPTLE), which we called TSB2. In designing a control peptide, TSB(Ctr) (RKKRRQRRR-A-ALVAYPLAHPTLA), we reasoned that if Glu were important for binding, then replacing Glu with Ala would result in a peptide that would fail to bind to hsp90 and, therefore, fail to impair vasodilation. To test these ideas, TSB2 and TSB(Ctr) (2.9 μM) were incubated with separate facialis arteries from healthy C57BL/6 male mice, whose vasodilation is mediated 100% by eNOS (3, 4, 9). Ten min later, excess peptides were removed by changing the buffer, and ACh-dose response curves of the preconstricted and pressurized vessels were determined as before (3, 4, 9).

To determine whether TSB2 peptide blocks the direct actions of NO, vasodilation responsive curves to an NO donor, DETA-NONOate, were measured on preconstricted and pressurized facialis arteries from healthy C57BL/6 male mice. The vessels were preincubated with either TSB2 or TSBSCR (scrambled TSB2 peptide, RKKRRQRRR-A-ELVLEVPLEHPTLE) (2.9 μM) for 10 min before the addition of DETA-NONOate.

To determine chronic effects of the decoy peptide on vasodilation in mice ex vivo, we injected C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), male, 6 weeks) with TSB2 (1 mg/kg/day) for 2 weeks. Facialis arteries from these mice and PBS-treated C57BL/6 mice were isolated and examined for changes in ACh-dependent vasodilation as described above (3, 4, 9).

Effects of TSB2 on EC- and eNOS-dependent O2− Generation in Situ—To determine whether TSB2 altered O2− generation in native EC on vascular tissues, anesthetized C57BL/6 mice were sacrificed by exsanguination, and aortas were perfused in situ at a rate of 2 ml/min with MOPS buffer containing TSB2 (2.9 μM) and dihydroethidine (DHE, 10 μM) in the absence and presence of L-NAME (200 μM) for 10 min followed by washout of excess, unreacted DHE with MOPS buffer alone. DHE is a cell-permeable probe that upon reaction with O2− is converted to a fluorescent 2-hydroxyethidium (2-OH-E+) product that can be quantified by fluorescent microscopy or HPLC (2, 10, 11). Aortas were quickly excised and examined for nuclear 2-OH-E+ staining (an index of O2− generation) using confocal fluorescent microscopy as previously described (2, 10). The intensity of the staining in each image taken from the confocal microscope was quantified by NIH image.
Determining Which Glutamic Acids and the Extent to Which These Glu Residues in eNOS Are Important for hsp90 Association—To determine which Glu residues are important to eNOS, we synthesized a series of decoy peptides that had Glu residues systematically replaced by Ala residues. The ability of these peptides to disrupt the eNOS-hsp90 interactions was analyzed by co-immunoprecipitation of eNOS as described before.

Site-directed Mutagenesis of eNOS and Establishing HEK-293 Mutant eNOS Cell Lines—The cDNA for bovine eNOS was mutated at 310, 314, 318, and 323 such that when translated Glu residues systematically replaced by Ala residues. Four glutamic acid site mutations of eNOS (E310A, E314A, E318A, and E323A) were generated by overlap extension PCR as described by Ful-Vasquez-Vivar et al. (14). Briefly, WT-eNOS and eNOS-4A HEK-293 cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HEPES, pH 7.4, containing either nothing else or 0.2 mM CaCl2, 0.2 M NADPH (final concentration), and A23187 (5 μM) with or without L-NAME (1 mM) at 37 °C for 30 min. The HEK cells cultures were washed 2× with Dulbecco’s PBS and then incubated in 2 ml of DMEM containing 2% fetal bovine serum, DHE (10 μM, final concentration), and A23187 (5 μM) for 30 min. The HEK cell pellet was lysed and sheared in 0.25 ml of Dulbecco’s PBS solution containing 0.1% Triton X-100 using a 0.5-inch 26-gauge needle on a 1-ml syringe. After mixing, an aliquot was removed for protein analysis. Next, 0.5 ml of n-butanol was added to the microcentrifuge tubes and vortexed vigorously for 15 min. The microcentrifuge tubes were centrifuged at 10,000 rpm for 10 min. An aliquot of 450 μl of the butanol phase was transferred to a new microcentrifuge tube and dried under a stream of argon gas at 37 °C. The dried residue was dissolved in water and quantified by HPLC (HP1100, Agilent Technologies) with fluorescence detection (excitation 510 and emission 595 nm) using authentic standards (2–10 μM) in the Free Radical Research Center as described (2, 10, 11). Care was taken to minimize exposure of reagents and samples to light during incubations and analysis.

Mice—Male C57BL/6 mice were purchased from The Jackson Laboratory. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

RESULTS

Effects of eNOS-derived Peptides on hsp90 Association with eNOS in EC Lysates—Incubation of the 12 eNOS-derived peptides individually with aliquots of lysates of proliferating EC cultures revealed where hsp90 bound to eNOS. Immunoprecipitation eNOS and Western blot analysis for eNOS and hsp90 revealed that B1, B2, B3, B4, and B5 all inhibited hsp90-eNOS interactions. B1 (291–310 aa), B2 (301–320 aa), and B3 (311–330 aa) significantly impaired association (Fig. 1, ~80%, p < 0.05, n = 6).

Effects of B2 on Basal and Stimulated eNOS-dependent -NO Generation and hsp90 Association with eNOS—Because B2 contained aa sequences that were common to B1 and B3, we examined the effects of B2 on stimulated EC -NO generation using the PTD peptide PEP1 to facilitate B2 uptake (for uptake efficiency, see the supplemental data). The B2:PEP1 mixture significantly inhibited basal and A23187-stimulated -NO production compared with BAEC cultures treated with PEP1 alone or nothing (control) (Fig. 2A, p < 0.05, n = 3). Immunoprecipitates of eNOS from these test groups reveal the B2:PEP1 mixture markedly decreased hsp90 association not only under basal conditions but also when the cultures were stimulated with A23187 (Fig. 2B, p < 0.05, n = 3). B2 decreased hsp90 associa-
tion to half that of the levels in BAEC cultures treated with PEP1 alone or nothing.

Effects of Acute Disruption of hsp90 Association on EC- and eNOS-dependent Vasodilation—To further pinpoint the region in eNOS that is responsible for association with hsp90, we redesigned a peptide from B1 to B3 regions in eNOS that contain the four Glu residues. This peptide, SB2, with the sequence of ELV-LEVPLEHPTLE, retained the ability to disrupt hsp90-eNOS interaction in BAEC lysates (Fig. 3A). Then a TAT PTD was added to SB2 to increase cellular uptake (for uptake efficiency, see supplemental data). The resultant peptide, TSB2, significantly decreased ACh-induced vasodilation by >50%, whereas the modified TSB(Ctr) had no effect on vasodilation compared with vehicle control (Fig. 3B, p < 0.02, n = 6). Failure of TSB2 to inhibit NO-mediated vasodilation from DETA-NONOate demonstrated that TSB2 disrupts cell signaling at a point upstream of NO (Fig. 3C, n = 4).

Effects of Chronic Disruption of hsp90 Association on EC- and eNOS-dependent Vasodilation ex Vivo—If hsp90 association with eNOS is important for vascular function in vivo, then chronic TSB2 treatments should inhibit EC- and eNOS-dependent vasodilation in the C57BL/6 mice. TSB2 treatments of C57BL/6 mice decreased ACh-induced vasodilation of facialis arteries (Fig. 3D, p < 0.01, n = 6). During isolation we noted that the facialis arteries from control C57BL/6 mice had thin layers of connective tissue on the advential side of the vessel. When facialis arteries from TSB2-treated mice were examined, connective tissue on the surface of the vessel appeared as large, thick fibers crisscrossing the adventitia (data not shown).

Effects of Disrupting hsp90 Association on eNOS-dependent \( \text{O}_2^\cdot \) Generation in Native EC—To test the hypothesis that disruption of hsp90 interactions with eNOS uncouples eNOS activity in native vascular EC, we perfused aortas of mice in situ with TSB2 and DHE under a physiological flow rate of 2 ml/min and then rapidly removed the vessels and analyzed the fluorescent intensity in the native EC by fluorescent confocal microscopy. TSB2 markedly increases \( \text{2-OH-E}^\cdot \) staining, an index of \( \text{O}_2^\cdot \) generation (Fig. 4, upper right compared with upper left), by a mechanism that could be inhibited in part by \( \text{i-NAME} \) (Fig. 4, lower right). On the basis that \( \text{i-NAME} \) is a substrate-specific inhibitor that blocks both NO and \( \text{O}_2^\cdot \) generation from eNOS...
An eNOS-derived Decoy Peptide Inhibits Vasodilation

**FIGURE 3.** Disrupting hsp90 protein-protein interactions with eNOS inhibits ACh-induced vasodilation of facialis arteries in vitro and ex vivo but not NO-donor DETA-NONOate-induced vasodilation. A, the SB2 peptide (6.2 μM) disrupts eNOS interactions with hsp90 in lysates from proliferating BAEC. IP, immunoprecipitates; WB, Western blot. B, acute exposure (10 min) of isolated, pressurized facialis arteries to TSB2 (2.9 μM) markedly reduces ACh-dependent vasodilation compared with untreated vessels (p < 0.02, n = 6–10), whereas treatment with TSB(Ctr) control peptide with Glu to Ala substitutions had no effect on vasodilation. C, 10 min exposure of isolated, pressurized facialis arteries to TSB2 (2.9 μM) has no effect on DETA-NONOate-induced vasodilation compared with 2.9 μM TSBSCR-treated vessels (n = 4). D, C57BL/6 mice were treated with TSB2 (1 mg/kg) or PBS (100 μl) for 2 weeks. At the end of this treatment period facialis arteries from TSB2-treated and untreated C57BL/6 mice were isolated, pressurized, and examined for responses to ACh as previously described (9). This line graph shows that TSB2 treatments alter vascular responses to ACh. Not only is ACh-induced vasodilation reduced, but other mechanisms of vasodilation are developing in vessels from TSB2-treated mice based on the fact that l-NAME fails to reduce vasodilation to base line.

**FIGURE 4.** Disrupting hsp90 protein-protein interactions with eNOS uncouples eNOS activity in native EC on aortas in C57BL/6 mice. Representative color images of fluorescent confocal micrographs of perfused male C57BL/6 mouse aortas are shown. The perfusion buffer contains MOPS DHE in the control (Ctr) group, MOPS + DHE + l-NAME in the Ctr + l-NAME group, MOPS + TSB2 + DHE in the TSB2 group, and MOPS + TSB2 + DHE + l-NAME in the TSB2 + l-NAME group as described under “Experimental Procedures.” TSB2 (2.9 μM) markedly increases 2-OH-E<sub>2</sub> staining in the nuclei of native vascular EC on TSB2 aortas compared with the control aortas. Although l-NAME does not alter 2-OH-E<sub>2</sub> staining in EC on control + l-NAME aortas compared with the control aortas, l-NAME does decrease 2-OH-E<sub>2</sub> staining in native EC on TSB2 + l-NAME aortas compared with the TSB2 aortas. (n = 12).

**FIGURE 5.** Systematic substitutions reveal which Glu residues are important for eNOS-hsp90 interactions. Sixteen peptides (2.9 μM) with different Ala residues replaced for Glu residues, including TSB2 itself, were incubated with lysates from proliferating EC. eNOS-hsp90 interactions were analyzed by Western blot analysis of immunoprecipitates of eNOS. The position of Ala replacing Glu in each peptide is listed below each bar. Lysates without any peptide incubation (Ctr) or with TSBSCR peptide were used as controls. The more Glu residues were replaced by Ala residues, the less influence the peptide had in disrupting eNOS-hsp90 interactions. (n = 3).

**Table 1.**

| Peptide | WT-eNOS Clone 2 | WT-eNOS Clone 3 | WT-eNOS Clone 4 |
|---------|-----------------|-----------------|-----------------|
|         |                 |                 |                 |
| Glu310  | E               | E               | E               |
| Glu314  | E               | E               | E               |
| Glu318  | E               | E               | E               |
| Glu323  | E               | E               | E               |

WT-eNOS clone 2 (Fig. 6A). On the basis of similar protein levels, these clones were selected for further analysis of eNOS-hsp90 association, eNOS-CaM association, NADPH consumption, and stimulated NO and O₂<sup>·</sup> generation. WT-eNOS and
An eNOS-derived Decoy Peptide Inhibits Vasodilation

FIGURE 6. Mutations E310A, E314A, E318A, and E323A in eNOS disrupt eNOS-hsp90 interactions and switch the enzyme from an NO synthase into a O\textsubscript{2}\textsuperscript{-}-generating NADPH oxygenase. A, wild type eNOS-transfected HEK-293 cells clone 2 shows similar expression levels of eNOS as mutant eNOS-4A transfected HEK-293 cells clone 4. WT, HEK-293 cells transfected with WT-eNOS; MT, HEK-293 cells transfected with mutant eNOS-4A; P, HEK-293 cells without transfection. B, mutant eNOS-4A consumes NADPH at the same rate as wild-type eNOS. WT, wild-type eNOS; MT, eNOS-4A; NS, not significant. C, mutant eNOS-4A binds CaM at the same level as wild-type eNOS when activated by A23187 (5 μM). WT, wild-type eNOS; MT, eNOS-4A. IP, immunoprecipitate; IB, immunoblot; D, mutant eNOS-4A does not associate with hsp90. Cell lysates were prepared from the unstimulated and A23187-stimulated (5 μM) cells expressing WT-eNOS and mutant eNOS-4A. eNOS was immunoprecipitated, and the levels of hsp90 association were determined. The mutant eNOS-4A binds little hsp90 in the basal or stimulated states, whereas WT-eNOS binds hsp90 at low levels under basal conditions and at high levels under stimulated conditions. E, mutant eNOS-4A loses the ability to generate NO upon A23187 stimulation (5 μM). Mock, HEK-293 cells transfected with empty vector. MT, HEK-293 cells transfected with eNOS-4A. WT, HEK-293 cells transfected with wild-type eNOS. The stimulated nitrate value was calculated by subtracting the basal nitrate value from the A23187-treated nitrate value (basal nitrate value is 0.44 ± 0.07 from mock, 0.36 ± 0.13 from MT, and 0.98 ± 0.34 nmol per mg of protein from WT. A23187-stimulated nitrite production by HEK-293-eNOS-4A cells (MT) is equivalent to that produced by mock cells. In contrast, HEK-293-WT-eNOS cells (WT) generate high level of nitrite upon A23187 stimulation.

DISCUSSION

Here we report that hsp90 associates with eNOS at 310–323 in eNOS. This critical interaction site was identified using eNOS-derived decoy peptides and site-directed mutagenesis. We have shown that both small peptides derived from eNOS and site-specific Glu to Ala mutations in eNOS can disrupt chaperone-dependent signaling with eNOS to inhibit eNOS-dependent NO generation and, in the case of peptides, also vasodilation by a mechanism that actually changes this enzyme function from an NO synthase into a O\textsubscript{2}\textsuperscript{-}-generating NADPH oxygenase.

One of the first pieces of evidence indicating the importance of 310–323 in eNOS was the fact that B1, B2, and B3 are all capable of disrupting hsp90 association with eNOS in cell lysates. We used cell lysates from proliferating BAEC to screen the eNOS-derived peptides for two important reasons. First, proliferating BAEC are known to possess a high level of hsp90 association with eNOS that is essential for maintaining a high level of BAEC NO generation and EC proliferation (3). Second, conducting the studies with lysates from proliferating BAEC rather than intact EC cultures removes confounding variables of cell physiology and metabolism. Thus, findings from these studies are justifiably restricted to protein-protein interactions.

After determining where hsp90 bound to eNOS, we reasoned that it might be advantageous to reduce the size of the B2 peptide and include a PTD to improve cellular uptake. TAT, a protein transduction domain of human immunodeficiency virus Tat protein, has been used to increase translocation of other small decoy peptides for inhibiting NADPH oxidoreductase activity in vascular tissues (6). In addition, it has been reported that TAT facilitates translocation of peptides or proteins through a variety of cells (20–23). Thus, we decided to use TAT as the PTD for generating decoy peptides that would be rapidly taken up.

Although B1, B2, and B3 all inhibit hsp90-eNOS association, there was no significant difference in the levels of inhibition among the three decoy peptides. It is interesting to note that SB2 (aa 310–323 of bovine eNOS) has an amino acid sequence that is highly conserved among different species including bovine, human, and mouse. Protein sequence alignment analysis by ClustalW revealed that the 14 amino acids in SB2 region of eNOS are almost identical among the three species, with only 1 amino acid difference. The second amino acid in SB2, which is
amino acid 311 in bovine, amino acid 318 in mouse, and amino acid 309 in human, is Leu in bovine and human but Met in mouse. The high level of conservation in the SB2 region indicates the importance of this binding domain to eNOS activity and function.

According to the three-dimensional structure of the dimer of the arginine oxygenase domain of eNOS (Protein Data Bank code 1d0c), the SB2 region is exposed to the aqueous environment and is composed of a short helix followed by a short \( \beta \)-pleated sheet, which terminates in another short helix on the crown of the arginine oxygenase domain (Fig. 8). This location likely provides ample access for the chaperone to modulate enzyme function. The calmodulin binding domain is located at aa 490–510 (24). This is sufficiently distant from the SB2 region that it is unlikely to interfere with calmodulin uptake or activity of eNOS. Likewise, the SB2 region is \(~535 \text{ aa from the autoinhibitory loop (aa 830–870)} (25). Taken together these reports (24, 25) and our data suggest that the SB2 region represents a novel site for post-translational regulation of enzyme function by hsp90.

We observed that acutely disrupting hsp90 association with eNOS inhibited eNOS-dependent vasodilation \textit{in vitro} (i.e. isolated vessels). Interestingly, chronic inhibition of hsp90 association with eNOS \textit{in vivo} also impaired eNOS- and EC-dependent vasodilation \textit{ex vivo} (i.e. isolated vessels from a treated mouse). Because TSB2 induces eNOS to switch from generating NO to \( \text{O}_2^\cdot \) (Fig. 4), a free radical whose physiological effects are diametrically opposed to those of NO, disrupting hsp90 association, should have profound physiological effects on vascular function as we showed in Figs. 3, A and C.

The fact that substituting Ala residues for Glu residues in TSB2 prevented the peptide from inhibiting hsp90 association and impairing vasodilation (Figs. 3A and 5) underscores the importance of the Glu residues at 310–323 in eNOS to this critical protein-protein interaction. Systematic replacement of Ala residues for Glu residues in TSB2 reveals Glu-314, Glu-318, and Glu-323 exert the greatest influence with Glu-310 exerting the least influence (Fig. 5). The importance of this domain to eNOS function was confirmed in the mutant eNOS-4A studies (Figs. 6–7). Results from these experiments not only show where the hsp90 binding domain is located on eNOS but also which Glu residues are important in regulating eNOS hsp90 protein-protein interactions.

Glu to Ala mutations of eNOS at 310, 314, 318, and 323 induce a major change in electrostatic charge in eNOS. Because loss of four Glu residues decreases the overall negative charge of the region, we cannot exclude the possibility that the loss of four Glu residues may directly alter the balance of NO and \( \text{O}_2^\cdot \) generation from eNOS-4A in addition to attenuating interactions with hsp90. However, having said this, it should be

FIGURE 7. Differential effects of L-NAME reveal the mutant eNOS-4A generates \( \text{O}_2^\cdot \) upon activation. L-NAME (1 mM) reduces \( \text{O}_2^\cdot \) production from A23187-stimulated (5 \( \mu \)M) mutant eNOS-4A-transfected HEK-293 cells but increases \( \text{O}_2^\cdot \) production in A23187-stimulated WT-eNOS-transfected HEK-293 cells. MT, HEK-293 cells transfected with eNOS-4A. WT, HEK-293 cells transfected with wild-type eNOS. (n = 8) A, superoxide anion generation in terms of 2-OH-E\(^{\cdot}\) (pmol/mg protein) from mutant eNOS-4A and wild-type eNOS-transfected HEK-293 cells stimulated with A23187 and treated with or without L-NAME. B, relative changes in superoxide anion generation = (A23187 + L-NAME) – (A23187).

FIGURE 8. Three-dimensional structure of eNOS showing the SB2 region and Glu-310, Glu-314, Glu-318, and Glu-323. SB2 region resides in the water interface of the crown of the arginine oxygenase domain. The three-dimensional structure of the dimer of the arginine oxygenase domain of eNOS is from Protein Data Bank code 1d0c. The protein structure is shown in ribbon style, whereas the heme molecules are shown in atoms and bonds. With the exception of the SB2 region, conformations are represented in different colors; helixes are in light green, the \( \beta \)-strand is in medium slate blue, the sharp turn is in sky blue, and the coil region is in light coral. The SB2 regions are colored as yellow with the four glutamic acids at 310, 314, 318, and 323 in red.
An eNOS-derived Decoy Peptide Inhibits Vasodilation

noted that our data clearly indicate TSB2 disruption of hsp90 interactions with WT-eNOS was sufficient to uncouple eNOS activity. Moreover, the substitution of Ala for Glu does not inhibit the ability of the enzyme to bind CaM and consume NADPH.

Exactly how hsp90 interacts with eNOS to modulate enzyme function is unclear. However, because chaperones are involved in changing protein conformation, hsp90 may hold eNOS in a conformation that favors NO production, whereas its absence allows the enzyme to take on a slightly different conformation that favors O$_2^-$ generation upon activation. In this way the ability to modulate a full range of enzyme. Indeed, eNOS has been shown to generate NO, O$_2^-$, and H$_2$O$_2$ and, with NO reacting with O$_2^-$ even peroxynitrite (17). It is interesting to speculate that the ability of eNOS to generate such an array of reactive oxygen species may provide unique advantages in regulating EC functions in response to physiological and pathophysiological stimuli.

In summary, we have shown that aa 310–323 in bovine eNOS, especially Glu-310, -314, -318, and -323, are critical sites for hsp90-eNOS interactions and coupled eNOS activity. Peptide sequences derived from eNOS can be used to design specific decoy peptides for studying the cell biology of this unique protein-protein interaction. Finally, our findings may have importance for understanding the role of other chaperone proteins and how their interactions with eNOS may influence vascular function.

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