

Heat Shock Protein 90 in Endothelial Nitric Oxide Synthase Signaling

Following the Lead(er)?

J.-L. Balligand

Endothelial nitric oxide synthase (eNOS) produces NO (and/or other reactive nitrogen species) in vascular endothelial cells and cardiomyocytes in response to a variety of agonists and mechanical stimuli (eg, stretch, shear). The central role of NO in the homeostatic balance of the healthy endothelium justifies a growing interest in the molecular mechanisms governing its production by eNOS in a stimulus- and (sub)cellular-specific fashion, including in response to a variety of drugs (eg, statins, angiotensin-converting enzyme inhibitors, β -blockers) widely used for the treatment of cardiovascular diseases. In addition to transcriptional regulation, eNOS activity is regulated posttranslationally by the availability of its substrate, L-arginine, and cofactors (eg, tetrahydrobiopterin [BH₄]), as well as protein-protein interactions with a number of partners that activate or inhibit the enzyme. Because new information on transient clustering of signaling molecules is likely to accumulate, especially with the use of functional proteomics,¹ thereby potentially increasing the list of eNOS regulators, one may legitimately ask these fundamental questions: (1) how do these different proteins associate with each other in a discrete subcellular compartment and in what sequential order and (2) how important is their (often transient) association for the proper functioning of any signaling pathway, especially in intact cells or in vivo. The availability of convergent data on x-ray crystal structure of the eNOS oxygenase domain, together with results using site-directed mutagenesis (or gene deletion experiments, eg, for caveolin-1 and caveolin-3) on several of the putative eNOS partners, now provides at least some of the answers and motivates the proposal of an updated model for eNOS activation.

Recap

Like the other two members of the NOS enzyme family, eNOS (1203 aa, 133-kDa protein encoded by *NOS3*, located on 7q35–7q36 of human chromosome 7) has a bidomain structure in which the N-terminal oxygenase domain, where

heme, BH₄, and L-arginine bind, is linked by a calmodulin-binding site to a C-terminal reductase domain containing binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and NADPH (see Figure). The association of eNOS into its active dimeric form is stabilized by heme and L-arginine as well as BH₄, the binding site of which is structurally maintained by a recently identified zinc center coordinated by critical cysteine residues. The N-terminal domain also contains the glycine residue supporting cotranslational and irreversible myristoylation, as well as cysteines (Cys15 and Cys26) reversibly and posttranslationally palmitoylated that are unique to eNOS and importantly condition its caveolar association.² When bound to its recognition site upon increases in calcium concentration, calmodulin increases the rate of electron transfer from NADPH to the reductase domain flavins and from the reductase domain to the heme center. The crystal structure of the reductase domain has not been solved yet, but the primary structure of eNOS (and neuronal NOS [nNOS]), unlike inducible NOS (iNOS), contains a 40 to 50 aa insert in the middle of the FMN-binding domain that may function as an autoinhibitory loop by destabilizing calmodulin binding at low calcium levels. Conversely, stimulus-induced increases in calcium may promote the displacement of the autoinhibitory loop upon binding of calmodulin to its binding site. Accordingly, inhibition of eNOS activity by calmodulin inhibitors and calcium removal justifies the traditional definition of eNOS as a calcium-sensitive enzyme.

eNOS is regulated by phosphorylation on serine, and, in specific circumstances, also on tyrosine and threonine residues. Stimuli such as vascular endothelial growth factor (VEGF), insulin, or shear stress induce phosphorylation at serine 1177 (for human)/1179 (for bovine eNOS) through phosphatidylinositol-3-kinase-dependent activation of Akt (protein kinase B), with an ensuing increase in enzyme activity that can be reproduced with recombinant Akt in vitro or overexpressed, constitutively active Akt in intact cells.^{3,4} Akt-phosphorylated eNOS, or eNOS where serine 1177 is mutated to aspartate (S1177D, which mimics the negative charge conferred upon phosphorylation), retains enzymatic activity despite low levels of calcium or calmodulin both in vitro and in situ. The mechanism for this apparent decrease in calcium sensitivity is unclear. Displacement of an autoinhibitory carboxy tail and influence on the more N-terminal autoinhibitory loop (see above) have been proposed as potential explanations, without eliminating the possibility of phosphorylation-induced changes in interaction with eNOS-associated proteins (see below), at least in intact cells. Other

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

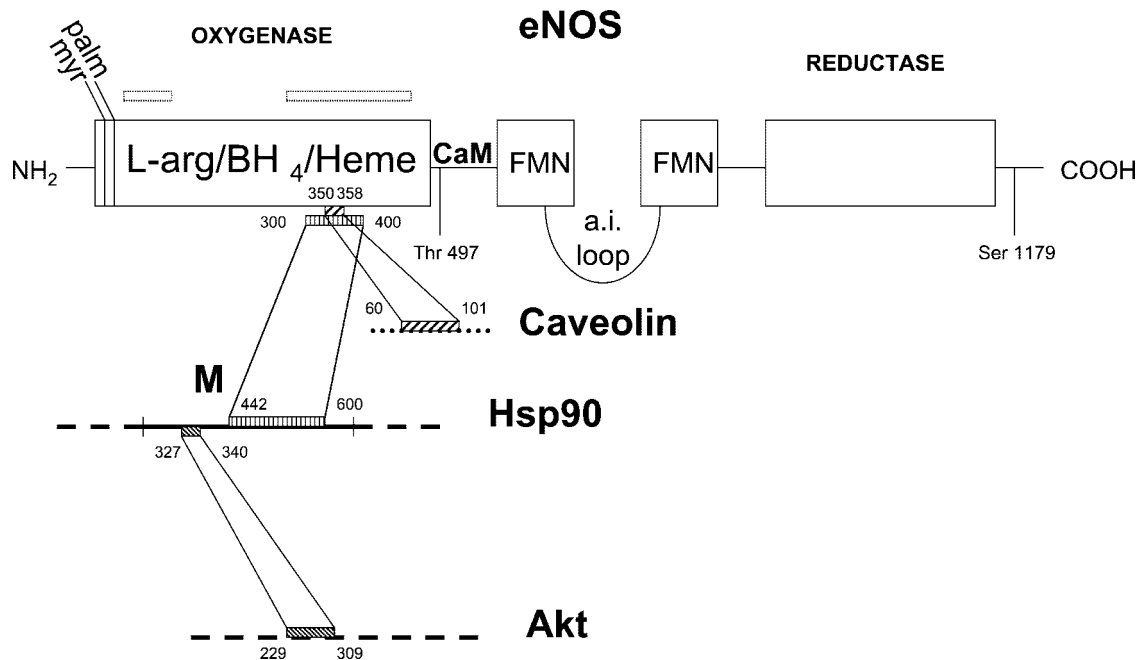
From the Unit of Pharmacology and Therapeutics, Department of Medicine, University of Louvain Medical School, Brussels, Belgium.

Correspondence to J.-L. Balligand, Unit of Pharmacology and Therapeutics, FATH 5349, Department of Medicine, University of Louvain Medical School, 53, Ave E Mounier, 1200 Brussels, Belgium. E-mail Balligand@mint.ucl.ac.be

(Circ Res. 2002;90:838–841.)

© 2002 American Heart Association, Inc.

Circulation Research is available at <http://www.circresaha.org>
DOI: 10.1161/01.RES.0000018173.10175.FF



Domain structure of bovine eNOS and mutual binding sites for caveolin, hsp90, and Akt. Oxygenase and reductase domains of eNOS are in solid boxes. Calmodulin (CaM) binding site, autoinhibitory loop, and sites for myristoylation and palmitoylation are indicated, as well as phosphorylation sites on threonine 497 and serine 1179. Potentially overlapping binding sites for caveolin and hsp90 on the C-terminal half of the oxygenase domain are indicated, as well as domains for dimerization (white boxes, above). The amino acid residue number at the start/end of each site for mutual protein-protein interaction is shown. L-arg indicates L-arginine; BH₄, tetrahydrobiopterin; FMN, flavin mononucleotide; and M, middle portion of hsp90.

kinases, eg, protein kinase A, protein kinase G, and AMP-activated kinase, also phosphorylate eNOS on serine 1177, the latter inducing also phosphorylation on threonine 495, as does protein kinase C. Phosphorylation on threonine 495 inactivates eNOS, so that dephosphorylation at this residue (eg, in response to stimulation with histamine or bradykinin), together with the activating phosphorylation on serine 1177, may coordinately regulate overall enzyme activity.⁵

Protein Partners

Caveolin

eNOS directly interacts with recombinant caveolin-1, or GST-caveolin fusion proteins in vitro, is coimmunoprecipitated with anti-caveolin-1 antibodies from endothelial cell extracts (and anti-caveolin-3 antibodies in extracts of cardiomyocytes), and colocalized with caveolin in plasmalemmal preparations enriched in caveolae. This interaction results in eNOS inhibition, which can be reversed by addition of exogenous calmodulin, suggesting a reciprocal regulation of the enzyme by inhibitory caveolin versus activating calcium/calmodulin.⁶ A consensus binding motif for caveolin is found within aa 350 to 358 of eNOS. Its functional importance was demonstrated by the inability of overexpressed caveolin to inhibit eNOS mutated on this motif.⁷ Conversely, the caveolin clamp on eNOS is mimicked by caveolin scaffolding domain (CSD) peptides comprising aa 82 to 101 in the primary binding region of caveolin-1 for eNOS (aa 60–101). Interaction with caveolin both maintains eNOS in a resting, inactivated state and ensures its proper localization to plasmalemmal caveolae (supported also by the enzyme prenyla-

tion), which conditions its activability by other signaling proteins (eg, G protein-coupled receptors [GPCRs]) concentrated in the same locale, although the proportion of total cellular eNOS targeted to caveolae may vary among (even endothelial) cell types. The functional relevance of the caveolin clamp in vivo was recently demonstrated from the hyporesponsiveness to constrictor agonists and enhanced vasorelaxation attributable to increased NO release in mice genetically deficient in caveolin-1.^{8,8a} Likewise, our group showed that statins potentiate eNOS activity by decreasing caveolin-1 abundance in vitro⁹ and in vivo (unpublished observations, 2002), at least in macrovascular endothelial cells where the caveolin pool is lower and the proportion of caveolin-bound eNOS is higher.

hsp90

This 90-kDa, mostly cytosolic, heat shock protein is expressed at high levels (accounting for up to 1% to 2% of total cellular protein content) even in unstressed conditions and is involved in the proper folding of specific protein substrates, in addition to its more recently apprehended role in the conformational regulation of signal transducing molecules, including members of the Src-kinase family of nonreceptor tyrosine kinases, Raf and other serine/threonine kinases, transcription factors such as steroid hormone receptors and p53, and eNOS, among others.¹⁰ hsp90 is an essential protein in eucaryotes, as illustrated by the lethality of homozygous disruption of its only homologue in *Drosophila* and disruption of many signaling pathways after heterozygous disruption. The two human gene products, hsp90 α and hsp90 β , share >85% sequence homology. hsp90 is endowed

with ATPase activity that is tightly coupled to movements of the protein, although the involvement of ATP in the mechanism of hsp90-assisted folding remains enigmatic. Nevertheless, the use of geldanamycin (GA) and related ansamycins that bind to the ATP site of hsp90 and disrupt its association with client proteins has helped to elucidate many of its functional implications in cell signaling.

hsp90 is associated with eNOS in the resting state, and, upon stimulation of endothelial cells with VEGF, estrogen, histamine, shear stress, and statins, the association between the two proteins is increased, concomitant with enhanced NO production.^{11,12} The mechanism of this activation remained unclear and could involve an allosteric modulation of eNOS resulting in enhanced affinity of calcium/calmodulin for the enzyme, as was demonstrated for nNOS.¹³ In this context, it is important to note that another client protein for hsp90 is Akt, the kinase involved in the activating phosphorylation of eNOS on serine 1177 (see above). Sato et al¹⁴ had already shown that hsp90 independently binds to and enhances the activity of Akt by preventing its dephosphorylation. Moreover, they had identified aa 327 to 340 of hsp90 β as critical for the binding to Akt, which involved aa 229 to 309 of the latter. Also, our group had previously demonstrated that stimulation of endothelial cells with VEGF or statins enhanced the recruitment, on the eNOS multiprotein complex, of phosphorylated Akt and hsp90; inhibition of this phenomenon with GA pointed to a key role for hsp90 as an adapter between its two client proteins, Akt and its substrate, eNOS.¹⁵

In the study by Fontana et al¹⁶ in this issue of *Circulation Research*, three different approaches are used to further characterize the domains of hsp90 required to bind eNOS, ie, yeast two-hybrid analysis, coprecipitation in cell extracts, and GST-fusion protein experiments in vitro. Previous analysis had implicated the C-terminus of hsp90 in the binding of proteins containing a tetratricopeptide domain (TPR; consisting of repeated degenerate motifs of 34 aa that share weak sequence homology), such as the immunophilins FKBP51 and 52. Whereas adenine nucleotides (and GA) bind to the ATP site, other protein partners, including Akt, bind in the middle domain (M domain) of hsp90. In the two-hybrid screen reported in the present study, the M domain (aa 442–600) likewise showed the strongest interaction with the N-terminal portion (aa 2–403) of eNOS (which also weakly interacted with more N-terminal sequences of hsp90). With this assay, more C-terminal sequences of eNOS (aa 304–709) showed no interaction with any hsp90 construct, thereby apparently restricting the relevant portions to aa 2 to 304 of eNOS. However, coprecipitation assays from extracts of cells transfected with epitope-tagged, full-length, or deleted constructs of both hsp90 and eNOS did not reveal an interaction between holo-hsp90 and a 1 to 300 aa fragment of eNOS. Instead, longer fragments, containing at least aa 1 to 400 did interact, leading the authors to propose regions between aa 300 and 400 of eNOS as functionally important for hsp90 binding. The reason(s) for this discrepancy remain(s) unclear. Nevertheless, GST-fusion proteins spanning N-, M-, or C-domains of hsp90 at least confirmed the preferential interaction of holo-eNOS with the M domain of hsp90.

The interesting result from these interaction analyses is that sequences of the M domain of hsp90 that bind eNOS (aa 442–600) or Akt (aa 327–340; see above) do not overlap, lending credence to the proposition that hsp90 serves an adapter for the kinase and its substrate, as mentioned above. Fontana et al¹⁶ further established that this mutual binding to the M domain not only allows Akt-dependent phosphorylation of eNOS, but even potentiates it (at least at early time points). By comparing the efficiency of phosphorylation of holo-eNOS versus a C-terminal NOS peptide containing serine 1179 (which would not bind to hsp90), they showed that this potentiation does not result from an intrinsic increase in kinase activity but probably results from a conformational adaptation of eNOS by hsp90 that renders it more phosphorylatable by Akt. Finally, the authors observed that overexpression of hsp90 in endothelial and COS-7 cells enhanced basal and VEGF-stimulated NO production, paralleled by increased phosphorylation of Akt and wild-type eNOS (but not the S1179A mutant, at least in COS cells), further emphasizing the functional importance of hsp90-modulated phosphorylation of eNOS on serine 1179 for its regulation. This also confirmed earlier results from our group showing increased recruitment of phosphorylated Akt, phosphorylated eNOS, and NO-dependent capillary tube formation in endothelial cells transduced with hsp90 constructs.¹²

These studies go to some length in answering the questions outlined in my introduction but do not resolve all of them. A close look at the primary sequence of eNOS (see Figure) mapping the regions where its partners interact suggests several overlaps for binding in the C-terminal half of the oxygenase domain, eg, for caveolin (aa 350–358) and hsp90 (aa 300–400). Although both proteins can be detected in eNOS immunoprecipitates at the same time (suggesting a binding equilibrium or additional nonoverlapping caveolin/hsp90 binding sites on eNOS), agonist stimulation clearly recruits more hsp90 on eNOS; not unexpectedly, this translates into less eNOS binding to caveolin, compatible with mutual and reciprocal binding to a common region. In support of this, CSD peptides completely abrogated the recruitment of hsp90, phosphorylated Akt, and the phosphorylation of eNOS in endothelial cells.¹² This then asks the question of the temporal sequence and the interrelationship between these events, especially in the context of the additional binding of calmodulin to its nearby binding site, which was also previously shown to be reciprocal with caveolin. Clearly, the coprecipitation methods used in the present study have their limitation in terms of temporal resolution. Nevertheless, our group has shown that VEGF stimulation produces an early (0.5-minute) disruption of the eNOS-caveolin complex, followed (at 2 minutes) by recruitment of hsp90 on eNOS. These early steps are clearly calcium-dependent and condition the recruitment of activated Akt to phosphorylate eNOS. Only when eNOS is phosphorylated does its activity become less calcium-sensitive.¹⁵ Does hsp90 itself displace caveolin from its overlapping binding site, or is this a consequence of the stabilization of calmodulin binding, itself favored by the subsequent phosphorylation of eNOS on serine 1177/(1179)? How does the inactivating phosphorylation of eNOS on threonine 495/(497) fit into this model, especially given the

surprising finding by Fontana and colleagues¹⁶ that hsp90 overexpression increases eNOS phosphorylation on this site? The notion that eNOS functions as a dimer adds another layer of steric complexity, given the identification of a dimer interface in the same C-terminal half of the oxygenase domain. Whether dimerization is properly taken into account in recombinant protein assays in vitro remains uncertain.

Another question voluntarily left aside, but equally important, is the relationship among these protein-protein interactions, changes in eNOS phosphorylation, and subcellular localization. In this context, it is worth recalling that the C-terminal half of the oxygenase domain contains overlapping sites for another protein partner of eNOS, NOSIP, which was shown to promote the translocation of eNOS to intracellular compartments.¹⁷ Finally, we still do not understand how agonists, physical forces, or drugs (eg, statins) promote the recruitment of hsp90 on eNOS. Some insights may be provided from further examination of the functional relevance of changes in tyrosine phosphorylation of hsp90,¹² now also emerging as a maestro of eNOS signaling.

References

- Gavin A, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature*. 2002;415:141–147.
- Feron O, Michel JB, Sase K, Michel T. Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. *Biochemistry*. 1998;37:193–200.
- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605.
- Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601.
- Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, Busse R. Phosphorylation of Thr⁴⁹⁵ regulates Ca²⁺/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res*. 2001;88:e68–e75.
- Michel JB, Feron O, Sase K, Prabhakar P, Michel T. Caveolin versus calmodulin: counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J Biol Chem*. 1997;272:25907–25912.
- Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S, Lisanti MP, Sessa WC. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin: functional significance of the nos caveolin binding domain in vivo. *J Biol Chem*. 1997;272:25437–25440.
- Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou H Jr, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem*. 2001;276:38121–38138.
- Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Haller H, Kurzchalia TV. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science*. 2001;293:2449–2452.
- Feron O, Dessy C, Desager JP, Balligand JL. Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. *Circulation*. 2001;103:113–118.
- Richter K, Buchner J. Hsp90: chaperoning signal transduction. *J Cell Physiol*. 2001;188:281–290.
- Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature*. 1998;392:821–824.
- Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O. Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. *Circ Res*. 2001;89:866–873.
- Song Y, Zweier JL, Xia Y. Heat-shock protein 90 augments neuronal nitric oxide synthase activity by enhancing Ca²⁺/calmodulin binding. *Biochem J*. 2001;355:357–360.
- Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A*. 2000;97:10832–10837.
- Brouet A, Sonveaux P, Dessy C, Balligand JL, Feron O. Hsp90 ensures the transition from the early Ca²⁺-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. *J Biol Chem*. 2001;276:32663–32669.
- Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, Tsuruo T, Sessa WC. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res*. 2002;90:866–873.
- Dedio J, Konig P, Wohlfart P, Schroeder C, Kummer W, Muller-Esterl W. NOSIP, a novel modulator of endothelial nitric oxide synthase activity. *FASEB J*. 2001;15:79–89.

KEY WORDS: nitric oxide ■ caveolin ■ heat shock protein 90 ■ endothelium