Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase

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Fleming, Ingrid, and Rudi Busse. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 284: R1–R12, 2003; 10.1152/ajpregu.00323.2002.—The endothelial nitric oxide synthase (eNOS), the expression of which is regulated by a range of transcriptional and posttranscriptional mechanisms, generates nitric oxide (NO) in response to a number of stimuli. The physiologically most important determinants for the continuous generation of NO and thus the regulation of local blood flow are fluid shear stress and pulsatile stretch. Although eNOS activity is coupled to changes in endothelial cell Ca²⁺ levels, an increase in Ca²⁺ alone is not sufficient to affect enzyme activity because the binding of calmodulin (CaM) and the flow of electrons from the reductase to the oxygenase domain of the enzyme is dependent on protein phosphorylation and dephosphorylation. Two amino acids seem to be particularly important in regulating eNOS activity and these are a serine residue in the reductase domain (Ser₁₁₇₇) and a threonine residue (Thr₄₉₅) located within the CaM-binding domain. Simultaneous alterations in the phosphorylation of Ser₁₁₇₇ and Thr₄₉₅ in response to a variety of stimuli are regulated by a number of kinases and phosphatases that continuously associate with and dissociate from the eNOS signaling complex. eNOS associated proteins, such as caveolin, heat shock protein 90, eNOS interacting protein, and possibly also motor proteins provide the scaffold for the formation of the protein complex as well as its intracellular localization.

calmodulin; caveolin; mRNA stability; phosphatase; serine phosphorylation; threonine phosphorylation

THE ENDOTHELIAL NITRIC OXIDE synthase (eNOS) is a constitutively expressed enzyme that oxidizes L-arginine to generate L-citrulline and nitric oxide (NO). The catalysis of this reaction requires a number of essential cofactors such as calmodulin (CaM), tetrahydrobioppterin (H₄B), flavin mononucleotide, FAD, and NADPH. The NO thus generated exerts a number of functions on the cardiovascular system. Acute activation of eNOS in blood vessels in response to the application of an agonist such as acetylcholine or bradykinin results in the activation of the soluble guanylyl cyclase in smooth muscle cells and the production of cGMP. An increase in intracellular cGMP levels may affect vascular tone by a number of mechanisms, for example by decreasing the intracellular concentration of free Ca²⁺ ([Ca²⁺]ᵢ; for review, see Ref. 80) as well as by activating protein kinase G and phosphorylating heat shock protein (Hsp) 20, which is reported to regulate force by binding to thin filaments and inhibiting cross-bridge cycling (10, 104, 105). A basal NO production or “vasodilator tone” can also be said to exist in vivo as the fluid shear stress generated by the flowing blood and pulsatile stretch of the vascular wall, as a consequence of the cardiac cycle, continually stimulate endothelial NO production. Although important for the regulation of blood flow, the continuous production of NO also helps to maintain the endothelium in an anti-atherogenic
state, in part by preventing the activation of transcription factors that determine the expression of proatherogenic gene products such as the adhesion molecules required for the attachment and sequestration of monocytes through the endothelial cell monolayer.

REGULATION OF eNOS EXPRESSION

Elucidation of the mechanisms and factors determining transcription of the eNOS gene under different physiological/pathophysiological conditions has long been considered central for a thorough understanding of alterations in vascular NO production. Although numerous studies have concentrated on the activity of the eNOS promoter and changes in eNOS mRNA expression, the stimuli associated with the most pronounced effects on eNOS protein levels (i.e., estrogen, TNF-α, and shear stress) are now appreciated to regulate posttranscriptional processes that mainly determine eNOS mRNA stability.

The eNOS Promoter

The eNOS promoter exhibits proximal elements such as Sp1 and GATA motifs but does not contain a TATA box, characteristics typical of a constitutively expressed gene (for reviews, see Refs. 39, 131). In addition, the human eNOS promoter possesses binding sites for numerous additional transcription factors, including activator protein (AP)-1, AP-2, Ets family members, myc-associated zinc-finger protein (MAZ), nuclear factor (NF)-1, NF-IL6, NF-κB, p53, PEA3, and YY1 as well as CACCC-, CCAAT-, heavy metal, acute phase response-, shear stress-, cAMP response-, retinoblastoma control-, interferon-γ response-, and sterol-regulatory cis elements. Deletion experiments revealed that some binding sites are essential for eNOS promoter activity, in particular Sp1 and GATA. The eNOS promoter also contains several half-palindrome motifs, for the estrogen- and glucocorticoid-responsive element (ERE andGRE), but no bona fide EREs or GREs (20, 48, 72, 78, 96, 123, 127, 132, 137).

Detailed dissection of the functionally important cis-DNA elements and the multiprotein complexes implicated in the cooperative control of the human eNOS gene in the vascular endothelium has highlighted the importance of two tightly clustered cis-regulatory regions (−104/−95 and −144/−115 relative to transcription initiation) in the proximal enhancer portion of the eNOS promoter (72). The nucleoprotein complexes that form on these regions in endothelial cells are reported to contain Ets family members, Sp1, variants of Sp3, MAZ, and YY1. Moreover, evidence has been presented for positive and negative protein-protein cooperativity involving Sp1, variants of Sp3, Ets-1, Elf-1, and MAZ. It therefore appears that the regulation of eNOS promoter activity in the vascular endothelium is highly complex and most probably tissue specific as it is determined by the binding of multiprotein complexes to the activator recognition sites (72).

Given the list of transcription factors that bind to the eNOS promoter it is hardly surprising that eNOS mRNA levels in cultured and native endothelial cells can be modulated by numerous stimuli. Estrogen is a good example of a hormone whose beneficial cardiovascular effects have been linked to an improvement of vascular function, mainly as a consequence of enhanced NO production. Numerous investigations have assessed the effects of estrogen on eNOS expression and, although 17β-estradiol has been reported by numerous investigators to upregulate eNOS mRNA and protein levels in cultured endothelial cells (74, 134), an equal number of investigators have found no convincing effect or even a decrease in eNOS expression (86). The best demonstrations of a link between estrogen and eNOS expression have been made using animal models, but even here chronic changes in estrogen levels have been reported to increase as well as decrease eNOS levels (66, 91, 100, 107, 125, 130).

Considerable effort has been made to determine whether or not common eNOS polymorphisms affect eNOS expression and/or activity in specific populations. Variants in the promoter region (none within any of the transcription factor binding sites identified to date) introns and exons have been identified and, although some are common and vary in different ethnic populations, the reported findings are largely inconsistent (for review, see Ref. 131).

The sequence in some important regions of the human and murine eNOS promoters is highly conserved (124), and using promoter-reporter insertional transgenic murine lines containing 5,200 base pairs of the native murine eNOS promoter directing transcription of a nuclear-localized β-galactosidase, it has been possible to map eNOS promoter activity in adult animals (125). Examination of β-galactosidase expression in the heart, lung, kidney, liver, spleen, and brain of such mice revealed a robust signal in large and medium-sized blood vessels. However, the eNOS promoter was apparently silent in arterioles, capillaries, and venules (125), a finding that may reflect the capability of smaller blood vessels in the microcirculation to regulate tone via NO-independent mechanisms.

Regulation of eNOS mRNA Stability

eNOS levels in endothelial cells can also be efficiently regulated by changes in eNOS mRNA stability. For example, eNOS mRNA levels are approximately four- to sixfold higher in proliferating than in confluent/growth-arrested endothelial cells (2), a difference that cannot be explained by differences in the eNOS transcriptional rate (114). Hypoxia and cytokines, such as TNF, also downregulate eNOS mRNA levels by decreasing the half-life of eNOS mRNA from 48 h under basal conditions to 3 h (92, 97, 135, 136).

Cell confluence, lipopolysaccharide, and cytokines affect eNOS mRNA half-life by a process involving the induction and expression of at least two cytosolic proteins [molecular mass 51 kDa (114) and 60 kDa (111)] that bind to a cytidine-rich region within the 3′-un-
translated region (3′-UTR) of the eNOS mRNA. By binding to the 3′-UTR, the protein probably alters its configuration, which renders it susceptible to RNase activity. There is at least preliminary evidence linking an increase in the binding of a 60-kDa protein to the 3′-UTR with a decrease in eNOS expression in hypercholesterolemic rabbits (67), and interference with the binding of the cytosolic proteins to the 3′-UTR of eNOS mRNA may be the mechanism underlying the increase in eNOS expression in endothelial cells treated with hydrogen peroxide (15, 30), ANG II (70, 98), estrogen (122), aspirin (3), and 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (54, 77, 88).

POSTTRANSLATIONAL REGULATION

Intracellular Translocation

It is now appreciated that the consequences of enzyme activation or the activation of signal transduction molecules can be determined to a large extent by the intracellular localization of the signaling complex. The monomers that compose the active eNOS dimer are myristoylated and palmitoylated (12, 109, 116) and thus can associate with intracellular membranes. This membrane association is required for the phosphorylation and activation of eNOS in response to stimuli such as vascular endothelial growth factor (VEGF; 40, 117). Functional eNOS can be detected in at least three membrane compartments, the plasma membrane (61), plasmalemmal caveolae (32, 44, 82, 118), and the Golgi apparatus (83, 99, 117). Both Golgi-associated and plasmalemmal eNOS are functional enzymes (40); however, the disruption of the Golgi apparatus does not affect the agonist-induced, endothelium-dependent, and NO-mediated relaxation of porcine coronary arteries, suggesting that the Golgi eNOS pool contributes little to the regulation of vascular tone (5).

The colocalization of the signal transduction molecules and proteins that comprise the “eNOS signaling complex” within the different membrane compartments facilitates enzyme activation, NO production, and the activation of downstream effector pathways. On the other hand, NO signaling in a compartment such as the caveolae can (at least at high concentrations) significantly modify the responses to other stimuli. For example, nitric oxide donors or analogs of cGMP inhibit the oligomerization of caveolin-1 and thus interfere with signaling via G protein-coupled receptors (79).

Although eNOS has been detected in the cytosol of some endothelial cells, it is unclear whether this eNOS is truly soluble or is still attached to a membrane fraction. For example, eNOS has been suggested to dissociate from the membrane (as a consequence of depalmitoylation) and be translocated to the cytosol in response to cell stimulation with high (receptor sequestration stimulating) concentrations of bradykinin (101, 106). However, this finding could not be confirmed by other groups (81) and may be an artifact resulting from the internalization of caveolae containing the B2 kinin receptor as well as eNOS (6, 68). eNOS has also been reported to translocate to a Triton X-100-insoluble/cytoskeletal cell fraction following stimulation with bradykinin (129) and tyrosine phosphatase inhibitors (35); again, this is not a universal observation. Although agonist-induced changes in the intracellular localization of eNOS remain controversial (121), the enzyme is thought to shuttle between different intracellular compartments in response to cell stimulation. Certainly estrogen activates the estrogen receptor-α and increases eNOS activity by a combination of its phosphorylation on Ser1177 and its subcellular translocation (18, 52, 60, 119). However, although it is tempting to speculate that each eNOS pool performs a distinct role, it is currently not possible to state definitively that the movement of eNOS from the caveolae to the peri-nuclear Golgi apparatus is particularly prominent after stimulation with agonists that modulate gene expression rather than NO production and vascular function.

Two groups have reported that eNOS can also be detected in the nucleus of endothelial cells (31) and brown adipocytes (50). Although the nuclear localization of eNOS may facilitate the interaction of NO with transcription factors, the conditions associated with a nuclear localization of eNOS and its exact role in this cell compartment remain to be determined.

Ca2+ and eNOS Activity

Classically, eNOS isoforms have been characterized on the basis of whether they are constitutively expressed and whether activation is dependent on an increase in [Ca2+]; and the binding of Ca2+/CaM to the enzyme. eNOS is constitutively expressed in most endothelial cells, particularly in conductance arteries and, of the three NOS isoforms, is the isoform that is most sensitive to changes in [Ca2+]. For example, when agonists such as acetylcholine or bradykinin are used to stimulate endothelial NO production, either the chelation of extracellular Ca2+ or the addition of a CaM antagonist abolishes NO production and endothelium-dependent relaxation (13, 85). Mechanistically, CaM binding to the CaM-binding motif is thought to displace an adjacent autoinhibitory loop on eNOS (and nNOS), thus facilitating NADPH-dependent electron flux from the reductase domain of the protein to the oxygenase domain.

eNOS can, however, be activated by certain stimuli without a sustained increase in [Ca2+]; being necessary; the most important of these stimuli is the fluid shear stress generated by the viscous drag of blood flowing over the endothelial cell surface. Shear stress, especially the application of flow to cultured endothelial cells or isolated vessels maintained for a time under no-flow conditions, can elicit Ca2+ transients (4, 65, 71). However, there is a discrepancy in the time course of the Ca2+ response and the time course of NO production, the former being transient, the latter maintained (4). Such observations led to the suggestion that a sustained increase in [Ca2+]i is not essential for the shear stress-induced activation of eNOS (4). The
application of fluid shear stress to endothelial cells results in the activation of the phosphatidylinositol 3-kinase and the subsequent activation of the serine kinases Akt and protein kinase A (PKA), which phosphorylate eNOS on Ser\(^{1177}\) and increase eNOS activity (28, 33, 51) (see below). This process has been referred to as the “Ca\(^{2+}\)-independent activation of eNOS”; however, the chelation of intracellular Ca\(^{2+}\) also abolishes the shear stress-induced increase in eNOS activity, suggesting that the increase in NO production is still strictly speaking Ca\(^{2+}\) dependent but that the enzyme can now be activated at resting Ca\(^{2+}\) levels (28). There are other stimuli (e.g., bradykinin and histamine) that also affect the phosphorylation of eNOS, but the signal transduction cascade activated requires an increase in [Ca\(^{2+}\)],. This apparent contradiction can be explained by the fact that the kinases that phosphorylate eNOS in response to shear stress or the application of a Ca\(^{2+}\)-elevating agonist are differentially sensitive to Ca\(^{2+}\). For example, the agonist-induced activation of the CaM-dependent protein kinase II (CaMKII) is highly dependent on an increase in [Ca\(^{2+}\)], whereas the shear stress-induced activation of Akt is not affected by Ca\(^{2+}\)-removal (28, 36).

**eNOS Phosphorylation**

eNOS can be phosphorylated on serine, threonine, and tyrosine residues (35), findings that highlight the potential role of phosphorylation in regulating eNOS activity. There are numerous potential phosphorylation sites, but most is known about the functional consequences of phosphorylation of a serine residue (human eNOS sequence: Ser\(^{1177}\); bovine eNOS sequence: Ser\(^{1179}\)) in the reductase domain and a threonine residue (human eNOS sequence: Thr\(^{495}\); bovine eNOS sequence: Thr\(^{497}\)) within the CaM-binding domain.

Ser\(^{1177}\). In unstimulated, cultured endothelial cells, Ser\(^{1177}\) is not phosphorylated but is rapidly phosphorylated after the application of fluid shear stress (28, 43), estrogen (76), VEGF (28, 95), insulin (73), or bradykinin (36). The kinases involved in this process vary with the stimuli applied. For example, shear stress elicits the phosphorylation of Ser\(^{1177}\) by activating Akt (28, 33) and PKA (9); estrogen and VEGF mainly phosphorylate eNOS via Akt; insulin seems to activate both Akt and the AMP-activated protein kinase (AMPK), whereas the bradykinin-induced phosphorylation of Ser\(^{1177}\) is mediated by CaMKII (36). When Ser\(^{1177}\) is phosphorylated, the flux of electrons through the reductase domain and, as a consequence, NO production are increased two- to threefold above basal levels (90).

Thr\(^{495}\). This residue is constitutively phosphorylated in all of the endothelial cells investigated to date (but not in COS cells transfected with a wild-type eNOS) and is a negative regulatory site, i.e., phosphorylation is associated with a decrease in enzyme activity. The link between phosphorylation and NO production can be explained by interference with the binding of CaM to the CaM-binding domain, and in endothelial cells stimulated with a Ca\(^{2+}\)-elevating agonist, such as bradykinin, histamine, or a Ca\(^{2+}\) ionophore, substantially more CaM binds to eNOS when Thr\(^{495}\) is dephosphorylated (36). The constitutively active kinase that phosphorylates eNOS Thr\(^{495}\) is most probably protein kinase C (PKC) (36, 89, 95), a finding that could account for the fact that protein kinase inhibitors and the downregulation of PKC markedly increase endothelial NO production (24, 64). The phosphatase that dephosphorylates Thr\(^{495}\) appears to be PP1 (see below).

Changes in Thr\(^{495}\) phosphorylation are generally associated with stimuli (e.g., bradykinin, histamine, and Ca\(^{2+}\) ionophores) that elevate endothelial [Ca\(^{2+}\)], and increase eNOS activity by 10- to 20-fold over basal levels. In response to such agonists, the activity of eNOS is not simply determined by the formation of a Ca\(^{2+}\)/CaM complex and its unregulated association with the enzyme, but rather by simultaneous changes in Ser\(^{1177}\) and Thr\(^{495}\) phosphorylation and resulting changes in the accessibility of the CaM-binding domain to CaM (Fig. 1). Stimulation of endothelial cells with growth factors/hormones such as estrogen does not appear to result in a change in the phosphorylation of Thr\(^{495}\), rather these agonists appear to increase NO production by two- to fourfold over basal levels by exclusively increasing the phosphorylation of Ser\(^{1177}\).

**Other Phosphorylation Sites**

Although the activation of eNOS is linked to simultaneous changes in the phosphorylation of Ser\(^{1177}\) and Thr\(^{495}\), there are certainly additional eNOS phosphorylation sites. Indeed, the eNOS immunoprecipitated from unstimulated cultured endothelial cells is serine phosphorylated (35, 94); however, the residue(s) that is constitutively phosphorylated under these conditions is not Ser\(^{1177}\).

Ser\(^{114}\) (bovine sequence Ser\(^{116}\)). This residue is localized within the oxygenase domain and was thought to be located within a consensus sequence for ERK1/2...
phosphorylation, although these MAP kinases do not seem to play a crucial role in the regulation of eNOS activity (see below). Bradykinin, the lipid signaling molecule lysophosphatidic acid (75), and fluid shear stress (43) have been reported to stimulate the enhanced phosphorylation of this amino acid, but the consequences of Ser114 phosphorylation on endothelial NO production remain to be elucidated. However, given the vicinity of Ser114 to the H4B binding site, it is tempting to speculate that this residue regulates the dimerization of eNOS by determining zinc binding or may act as a phosphoryl switch determining whether eNOS generates NO or superoxide anions (O$_2^-$). Ser633 (bovine sequence Ser635). Ser633 is located within the so-called auto-inhibitory loop, a peptide insert present in the Ca$^{2+}$-dependent NOS isoforms that is thought to be folded in such a way as to physically impede the access of CaM to its binding domain, thus throttling enzyme activity. Deletion of the auto-inhibitory loop increases the activity of the two Ca$^{2+}$-dependent NOS isoforms (128) and as this insert contains a number of phosphorylatable amino acid residues [12 of the 45 amino acids are either serine or threonine (110)], it is tempting to speculate that the phosphorylation may alter the conformation of the insert and thus the ability of CaM to access its binding site. Ser633 can be phosphorylated in vitro by PKA and PKG (14), but otherwise the functional relevance of Ser633 phosphorylation has not been addressed in detail in an intact cell system. Indeed, the few experimental studies that compared the potential of phosphorylation on Ser1177 vs. Ser633 in regulating eNOS activity concluded that Ser1177 played a major role in the regulation of NO production whereas either no Ser633 phosphorylation could be detected or no consequence of phosphorylation was evident (28, 41).

Tyrosine. Although eNOS can be tyrosine phosphorylated and endothelial NO production can be modulated by inhibitors of tyrosine kinases as well as tyrosine phosphatases (34, 35), almost nothing is known about the residues that are phosphorylated or the kinases that are involved. The nNOS can be activated by Src-homology tyrosine phosphatase-2-mediated dephosphorylation of tyrosine residues (21), but it is currently speculated that the tyrosine phosphorylation of eNOS is not likely to affect eNOS activity directly but more probably determines the docking of associated scaffolding and regulatory proteins (35, 62). Elaborating the functional consequences of eNOS tyrosine phosphorylation is hampered by the fact that this modification is only clearly evident in primary cultured cells (35, 45), and investigators using passaged endothelial cells have generally been unable to detect phosphorytrosine residues on eNOS (22, 94, 129).

eNOS-Associated Proteins

Concepts related to the regulation of intracellular signaling by alterations in the localization and association of distinct protein mediators are continually changing. Whereas the activation of eNOS has long been known to be dependent on protein-protein interactions, especially between CaM and eNOS, numerous additional eNOS-associated proteins have been identified over the last five years. It now seems that endothelial NO production is not simply dependent on the expression of the eNOS enzyme but is determined by an eNOS signaling complex that consists of the enzyme and a conglomerate of adaptor proteins, structural proteins, kinases, phosphatases, and potentially also motor proteins that affect complex association and determine intracellular localization.

Caveolin-1. The binding of caveolin-1 to a consensus site (K$_{350}^{	ext{R}}$SAAPSGW$_{358}$) in eNOS is proposed to antagonize CaM binding and thereby inhibit enzyme activity (for review, see Refs. 42 and 47). Indeed, coexpression of eNOS and caveolin-1 in COS-7 cells leads to a marked inhibition of enzyme activity (95), whereas a cell-permeable peptide harboring the scaffolding domain of caveolin-1 can inhibit NO-mediated vascular permeability and vasodilator responses in vivo (11). In aortas from caveolin knockout mice, on the other hand, the relaxant response to acetylcholine is enhanced and the constrictor response to phenylephrine blunted as a consequence of enhanced basal NO production (29, 103).

The caveolin-binding motif in eNOS lies between the heme and the CaM-binding domain adjacent to a glutamate residue, which is necessary for the binding of L-arginine, suggesting that caveolin-1 may interfere with heme iron reduction, similar to the L-arginine analogs (47). Other groups have, however, detected interaction between both the NH$_2$- and COOH-terminal domains of caveolin-1 and the oxygenase domain of eNOS (47, 69). In experiments to examine how a caveolin-1 scaffolding domain peptide would affect NO synthesis, it was demonstrated that caveolin-1 must bind to the reductase domain of eNOS to inhibit enzyme activity (49). Therefore, caveolin-1 binding to the reductase domain of eNOS compromises its ability to bind CaM and to donate electrons to the heme subunit, thereby inhibiting NO synthesis (49).

Although eNOS and caveolin are expressed throughout the arterial system (115), the majority of studies demonstrating eNOS-caveolin association were performed in subconfluent cells. Indeed, using immunohistochemistry, eNOS and caveolin appear to be concentrated along the leading edge of proliferating cells (44). In confluent cultured cells as well as in native endothelial cells, the majority of cellular eNOS is not colocalized with caveolin but is concentrated at cell-cell contacts in the vicinity of platelet-endothelial cell adhesion molecule 1 (PECAM-1) and within the Golgi apparatus (1, 55). Although both caveolin and eNOS can also be found in the Golgi apparatus, they are separated into distinct perinuclear compartments that behave differently in the presence of a microtubule-depolymerizing drug, thus indicating that these two proteins are not in direct physical contact and that eNOS activity is not regulated by caveolin-1 within the Golgi complex (56).
Some of the most interesting data relating to the regulation of eNOS by caveolin is related to the vascular effects of estrogen. Indeed, estrogen has been reported to markedly affect the formation of caveolae (126) as well as the expression of caveolin-1 (66). Chronic changes in estrogen status can differentially affect eNOS and caveolin-1 protein levels in native endothelial cells, i.e., eNOS levels go down and caveolin-1 levels go up (100). Manipulating the expression of either eNOS or caveolin-1 alone does not restore eNOS function in arterioles from estrogen-depleted rats and only the simultaneous upregulation of eNOS and downregulation of caveolin-1 has been associated with the normalization of activity (133).

**Dynamin.** As the concept that eNOS can shuttle between at least two intracellular pools gains acceptance, attention has begun to turn to the mechanism controlling this shuffling process and in particular to the association of eNOS with potential motor proteins. One motor protein, which affects the sequestration of G protein-coupled receptors, such as the muscarinic acetylcholine receptor (26), via caveolae and which can also modulate signaling pathways by means of direct protein interactions is dynamin (23, 63). Dynamin 2 can associate with eNOS, in the Golgi, and the association of the two proteins can be increased by Ca$^{2+}$-ionophores (16). Moreover, in both in vitro assays as well as cultured endothelial cells, an increase in dynamin levels results in enhanced eNOS catalysis (16). There is currently no information available regarding the potential role of dynamin in the physical transport of eNOS between different intracellular locations.

**G protein-coupled receptors.** There have been reports that eNOS associates directly with G protein-coupled receptors and specifically with the intracellular domain 4 of the B$_2$ kinin, the ANG II AT$_1$, and the endothelin ET B receptors (68, 87). eNOS and G protein-coupled receptors are reported to be associated in unstimulated cells, whereas dissociation of the proteins occurs in response to cell stimulation. Moreover, the association of eNOS with a fusion protein corresponding to intracellular domain 4 of the B$_2$ receptor is reported to inhibit enzyme activity (68). An inhibitory effect of a synthetic peptide based on intracellular domain 4 has also been observed using a purified nNOS (53). In the latter case, inhibition could not be attributed to an interference of the peptide with L-arginine or H$_4$B binding or to interference with the cytochrome c reductase activity of the enzyme. Rather, the binding of the B$_2$ kinin receptor peptide was reported to block flavin to heme electron transfer in nNOS (53). Reports of an inhibitory interaction between G protein-coupled receptors and NOS are, however, controversial, and there is currently no convincing evidence to suggest that this interaction occurs in native cells in situ.

**Hsp90.** The primary function of Hsp90 is its involvement in a multicomponent chaperone system that is responsible for the folding of proteins such as steroid receptors and cell cycle-dependent kinases (17). Hsp90 is involved in the folding of NOS enzymes and is reported to determine the insertion of heme into the immature protein (8). In addition to this function, Hsp90 can also act as an integral part of numerous signal transduction cascades.

Hsp90 can associate with eNOS in resting endothelial cells and endothelial cell stimulation with VEGF, histamine, fluid shear stress, and estrogen all enhance the interaction between Hsp90 and eNOS at the same time as increasing NO production (46, 108). Exactly how Hsp90 regulates eNOS activity has been relatively well elucidated and the association of Hsp90 with eNOS appears to be determined by the agonist-stimulated tyrosine phosphorylation of Hsp90 (58). In vitro characterization of the domains of Hsp90 required to bind eNOS revealed that the M region of Hsp90 interacts with the amino terminus of eNOS (amino acids 442–600) and Akt. Moreover, the addition of purified Hsp90 to in vitro kinase assays facilitated Akt-driven phosphorylation of recombinant eNOS protein suggesting that Hsp90 may function as a scaffold for eNOS and Akt. Additional effects cannot be ruled out because Akt activity is enhanced following binding to Hsp90, an effect that may be related to the protection against Akt dephosphorylation by the phosphatase PP2A (112). Thus, in response to endothelial cell stimulation, eNOS and Akt appear to be recruited to an adjacent region on the same domain of Hsp90, which facilitates eNOS phosphorylation and enzyme activation (37). Whether Hsp90 also acts as a scaffold for other eNOS-associated proteins remains to be determined. However, a recent study suggested that Hsp90 increases the affinity of nNOS for CaM (120), and experiments using eNOS have shown that Hsp90 facilitates the CaM-induced displacement of caveolin from eNOS (57).

To study the relationship between Hsp90-mediated signaling and NO production, extensive use has been made of the ansamycin antibiotic geldanamycin, which is assumed to be a specific inhibitor of Hsp90. However, recent studies have suggested that some of the observations made using this substance can be attributed to Hsp90-independent effects. For example, treatment of endothelial cells with geldanamycin results in a dramatic increase in O$_2^-$ generation, which is independent of eNOS activity (27). Similar effects have also been reported using purified nNOS (8). Moreover, geldanamycin directly oxidizes ascorbate, consumes oxygen, and decreases the bioavailability of NO generated by 3,4-dihydrodiazete 1,2-dioxide in smooth muscle cells, an effect that can be prevented by superoxide dismutase (27). The finding that geldanamycin can generate O$_2^-$ on its own may account for previous reports suggesting that Hsp90 may play a role in regulating eNOS uncoupling, i.e., the phenomenon by which eNOS generates O$_2^-$ rather than NO (102).

**Kinases.** Most of the kinases shown to phosphorylate eNOS on serine or threonine residues physically associate with the enzyme, either directly or via binding to an adaptor protein. Little is known about the mechanisms determining the association of eNOS with PKA (41), AMPK (19), or CaMKII (36), but the binding of Akt to eNOS is thought to be dependent on the associ-
ation of eNOS with Hsp90 (see above). Other kinases reported to affect eNOS activity are the MAP kinases, ERK1 and ERK2, as well as the cyclic nucleotide-dependent kinases PKA and PKG. Not much detailed information is available regarding the mechanisms by which these kinases regulate NO generation and some of the reports are controversial, because although eNOS in bovine endothelial cells was reported to be inhibited by ERK1/2 (7), inhibitors of ERK1/2 activation failed to affect the NO-mediated relaxation of isolated arteries or NO generation by porcine or human endothelial cells (36, 113). PKA and PKG, on the other hand, phosphorylate a purified recombinant eNOS protein on Ser1177 and Ser633 in an in vitro system (14). Additional mechanisms may also play a role in vivo as PKA may also stimulate the dephosphorylation of Thr495 by activating the phosphatase PP1 (95).

**eNOS interacting protein.** eNOS interacting protein (NOSIP) is a 34-kDa protein with unknown function that was identified using the yeast two-hybrid system. NOSIP binds to the carboxyl-terminal region of the eNOS oxygenase domain, and coimmunoprecipitation studies demonstrated that the interaction between eNOS and NOSIP in vitro as well as in cultured cells can be inhibited by a synthetic peptide corresponding to the caveolin-1 scaffolding domain (25). Functionally, NOSIP is reported to decrease eNOS activity and promote the translocation of eNOS from the plasma membrane to intracellular compartments (25). However, most studies have been performed using nonendothelial cell lines overexpressing both proteins. Therefore, the physiological role, if any, played by NOSIP in the regulation of eNOS activity remains to be determined.

**Phosphatases.** Because eNOS activity is regulated by phosphorylation, it is logical that alterations in phosphatase activity can also affect NO generation. Indeed, PP1 and PP2A play distinct roles in the regulation of eNOS phosphorylation (33, 36, 95).

PP1 dephosphorylates Thr495 and inhibition of PP1 results in the hyperphosphorylation of Thr495, which inhibits eNOS activity (36). The mechanism of PP1 activation by receptor-dependent agonists is still unclear, but after the application of bradykinin to endothelial cells, the dephosphorylation of Thr495 is a Ca2+-dependent process (36), whereas in forskolin-stimulated endothelial cells, the process is dependent on the activation of PKA (95). The fact that the bradykinin-dependent dephosphorylation of Thr495 is Ca2+-dependent initially suggested that this process should be regulated by a Ca2+-dependent phosphatase such as PP2B (calcineurin) (59). However, the calcineurin inhibitors, cyclosporin A and FK506 do not affect bradykinin-induced changes in Thr495 phosphorylation or bradykinin-induced NO production (36, 95).

PP2A is the phosphatase that dephosphorylates Ser1177 (33, 95) and PP2A inhibitors, such as okadaic acid, increase eNOS activity by two- to fourfold, i.e., to the same extent as stimuli such as fluid shear stress (33). PKC, which phosphorylates Thr495, can also promote the dephosphorylation of Ser1177 by regulating the activity of PP2A (36, 95). This dual function amplifies the PKC-mediated inhibition of eNOS activity. Both PP1 and PP2A are reported to associate with eNOS but this association is not modified by cell stimulation (95).

Nothing is known about the phosphatase(s) that regulate the tyrosine phosphorylation of eNOS. However, nNOS can be tyrosine phosphorylated and is reported to interact with SHP-1 (38, 84) and in response to cell activation, SHP-1 rapidly recruits nNOS and tyrosine dephosphorylates it, thus increasing enzyme activity.

**CONCLUSION**

The regulation of eNOS is a process determined by a cascade of events determining eNOS mRNA and protein levels, the formation of the eNOS signaling complex, its intracellular translocation, and eNOS phosphorylation. Although many of these steps have been relatively well elucidated in in vitro models and in cell lines overexpressing one or more components of the signaling complex, much more work is required to determine which modifications play a dominant role in the regulation of eNOS activity in vivo and how these steps are influenced by the pathophysiological changes that ultimately lead to “endothelial dysfunction” and to the development of atherosclerosis.

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