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Endothelial Caveolar Subcellular Domain Regulation of Endothelial Nitric Oxide Synthase

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SUMMARY

1. Complex regulatory processes alter the activity of endothelial nitric oxide synthase (eNOS) leading to nitric oxide (NO) production by endothelial cells under various physiological states. These complex processes require specific sub-cellular eNOS partitioning between plasma membrane caveolar domains and non-caveolar compartments.
2. eNOS translocation from the plasma membrane to intracellular compartments is important for eNOS activation and subsequent NO biosynthesis. We present data reviewing and interpreting information: 1) the coupling of endothelial plasma membrane receptor systems in the caveolar structure relative to eNOS trafficking; 2) how eNOS trafficking relates to specific protein-protein interaction for inactivation and activation of eNOS; and 3) how these complex mechanisms confer specific subcellular location relative to eNOS multi-site phosphorylation and signaling.
3. Dysfunction in regulation of eNOS activation may contribute to several disease states; in particular gestational endothelial abnormalities (preeclampsia, gestational diabetes, etc) that have life-long deleterious health consequences that predispose the offspring to develop hypertensive disease, type II diabetes and adiposity.¹

Keywords

Endothelium; nitric oxide; vascular; caveolae; Barker Hypothesis

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INTRODUCTION

Regulation of the activity of endothelial nitric oxide synthase (eNOS) within the endothelial cell is a complex process. This process has been shown to be closely associated with the enzyme's specific sub-cellular localization which partitions eNOS between the caveolar domain in the plasma membrane and the non-caveolar compartments comprising the cytoplasm and the microsome. Previous review articles have popularized a phrase coined in the real estate field "location, location, location". This phrase makes a specific point that several models of eNOS activation lead to elevations in NO production by the healthy endothelium. This phrase also incorporates the translocation of eNOS away from the plasma membrane and into the cell.² However, due to its complex regulation no one unifying model has been shown to describe all of these regulatory stages.

The purpose of this review is to present evidence from numerous studies including our own to better understand the endothelial caveolar regulation of endothelial nitric oxide synthase. Three areas will be discussed: 1) we show the coupling of endothelial plasma membrane signaling systems in the caveolar structure relative to eNOS trafficking, 2) we relate this trafficking to specific protein-protein interaction for inactivation and activation of eNOS, 3) we describe how trafficking and protein-protein interactions relate to eNOS multi-site phosphorylation.

Ultimately, the dysfunction of the eNOS activation and NO bioavailability negatively contribute to several endothelial abnormalities such as preeclampsia and gestational diabetes. Thus, understanding the complex endothelial-NO-derived vasodilation may provide possible targets to alleviate and possibly improve fetal growth and development in a compromised pregnancy.

CAVEOLAE AND THEIR ROLE IN TRAFFICKING OF eNOS

The trafficking and resident localization of eNOS into the caveolae has been established by several studies.^{2, 3} Caveolae are specialized regions of the plasma membrane that have a unique composition and function. Collectively, caveolae are considered to be a conglomerate of specialized endothelial cell plasma membranes facing the circulating blood. These important structures function as a focal transducer of hormonal and mechanical signals for eNOS activation and elevations in NO biosynthesis. Caveolae are Ω -shaped invaginations that measure 50–100nm and are constructed in such a way to form their structure by polymerization of their primary scaffolding proteins caveolin (cav)-1, cav-2 and cav-3. Within the cardiovascular system, cav-1 and cav-2 are expressed in endothelial cells with cav-1 being the predominant isoform. By contrast, cav-3 is the only isoform expressed in cardiomyocytes, visceral-smooth muscle and skeletal muscle cells.^{4, 5} These specialized regions act to reduce the fluidity of the plasma membranes that they segregate.⁶ Using transmission electron microscopy, we show in Figure 1 the overall shape and location of caveolae in ovine uterine artery endothelial cells (UAECs) and are identified by the arrows. Caveolae are also rich in glycosphingolipids, sphingomyelin and lipid-anchor membrane proteins (known as GPIs). Other components found in the caveolae include non-receptor tyrosine kinases, non-receptor ser/thr kinases, G protein-coupled receptors, G-proteins,

enzymes, GTPases and cellular proteins/adaptors.⁷⁻⁹ In Table 1 we present the list of known proteins and their functions that are found in the caveolae. The protein interactions observed within the caveolae domain facilitate both regulatory mechanism and signaling propagation. Thus a more comprehensive understanding of caveolae composition and function is still necessary in order to determine their role in cardiovascular diseases.

Structurally, cholesterol, glycosphingolipids and sphingomyelin form the lipid core in caveolae; which is referred to as the β phase. This β phase is defined as an interphase of aqueous vs. lipid soluble substances within the lipid bilayer. Functionally, this caveolar core is important for attracting and trafficking lipid-modified proteins to these specialized plasma domains, listed in Table 1. These lipid modifications are covalent attachments of long-chain acyl groups (myristate and palmitate) that change the hydrophobic character of proteins. The new hydrophobic character of these proteins then aids in their anchoring to the β phase within caveolae and in some cases other regions of the plasma membrane.¹⁰ This compartmentalization and centralization of all these proteins, receptors and enzymes is thought to help establish an efficient cellular response to endocrine, paracrine and autocrine signals. Additionally, it has been shown that hemodynamic factors (i.e. laminar shear stress) affect the endothelium.¹¹⁻¹⁶

Shear stress has been shown to have direct and indirect effects on the endothelium. The direct effect of shear stress mediated by the endothelium is the reduction in vascular tone that occurs within seconds or minutes. This acute process acts as a physiological servo-mechanism for sensing rapid changes in blood flow, which in turn returns shear stress back to a set-point for a healthy endothelium.¹¹ This process has been termed “normalization of shear.” An indirect effect of laminar shear stress is observed through the increase or decrease in blood flow. The change in blood flow in turn changes the relative agonist(s) concentration of the microenvironment on the endothelial cell surface. The changes in the agonist concentrations at the cell surface thus influences receptor-ligand interactions at the plasma membrane and/or the caveolae consequently altering the cellular response to the agonist.¹¹

The control of eNOS activation falls under a very complex regulatory mechanism that includes tonic inhibitory interaction with cav-1, post-translational modifications including myristoylation, palmitoylation and phosphorylation events, as well as stimulatory responses to rises in intracellular calcium concentrations. Figure 2 summarizes the different levels of regulation known to affect eNOS enzymatic activity. The protein eNOS contains a reductase and an oxygenase domain and between them is a Ca^{2+} -calmodulin (CaM) binding domain.¹⁷ The reductase domain also houses the nicotinamide adenine dinucleotide phosphate (NADPH) binding domain close to the C-terminus, flavin adenine dinucleotide (FAD), and the flavin mononucleotide (FMN) close to the CaM binding domain.¹⁷ During post-translational modification events, the fatty acid moieties are added within the N-terminus of eNOS with one myristoylation site at glycine 2 and two palmitoylation sites at cysteine 15 and 26.¹⁸ Shaul *et. al.* methodically identified that myristoylation alone aids in targeting eNOS to the caveolae at a 10-fold higher rate than the two palmitoylation events, however, both types of fatty acid modifications will target eNOS to the caveolae.¹⁸ Palmitoylation of eNOS has also been shown to be a dynamic and reversible process, thereby forming the

eNOS complex that makes up this caveolar regulatory system. Moreover, the addition or removal of phosphate (PO_4^{3-}) groups have also been shown to increase or decrease eNOS activation state as described in detail below. For example as shown in Figure 2, the primary phosphorylation sites known to have functional effects on eNOS are ser 1177 ($\text{ser}^{1177}\text{eNOS}$) located in the FMN binding domain, ser 635 ($\text{ser}^{635}\text{eNOS}$) located within the NADPH binding domain, thr 495 ($\text{thr}^{495}\text{eNOS}$) located in the CaM binding domain, and ser 116 ($\text{ser}^{116}\text{eNOS}$) located in the oxygenase domain.^{12, 17, 19–22} Laminar shear stress and multiple calcium mobilizing agonists have shown to increase NO production that follows a rise in intracellular calcium, thus making this process Ca^{2+} -dependent.^{12, 23, 24} However, others have shown that eNOS activation is regulated by an intracellular Ca^{2+} -independent process.^{21, 22, 25} For example, Boo *et. al.* showed an increase in $\text{ser}^{1177}\text{eNOS}$ and $\text{ser}^{635}\text{eNOS}$ phosphorylation with a concurrent decrease in $\text{thr}^{495}\text{eNOS}$ phosphorylation when bovine aortic endothelial cell (BAEC) were transfected with a constitutively active PKA catalytic subunit. In this same study, they also reported an increase in NO production in the presence of increasing concentration of a calcium chelator, BAPTA-AM.²²

Agonist-receptor activation increases eNOS activity and is associated with the same mechanism that translocates the enzyme away from the plasma membrane.^{26, 27} Several important post-translational modifications are needed to adequately and efficiently stimulate NO production. Studies that utilized an over-expression of cav-1 have showed a reduced basal NO production in a “control” cellular state, cav-1 interaction with eNOS has been shown to negatively regulate NO release.⁴ It has been established that eNOS requires palmitoylation and myristoylation in order to be targeted to the caveolae microdomains. Upon agonist activation, (e.g. ATP, Bradykinin, etc.), eNOS translocates away from caveolae, thus removing the tonic cav-1 inhibition.^{3, 9} Feron *et. al.* demonstrated that the palmitoylation of eNOS may also be involved in its translocation process.³ In this study, they identified that after agonist-dependent eNOS activation, the removal of the tonic inhibition between eNOS and cav-1 coincides with de-palmitoylation concomitant with the observed translocation of eNOS to the non-caveolar fraction; which is indicative of increased NO biosynthesis. Conversely, when eNOS returns to the membrane/caveolae, it is re-palmitoylated and its inhibitory interaction with cav-1 is reasserted.³ In contrast to the inhibitory effect of cav-1 on eNOS, Heat Shock Protein (HSP) 90 is thought to play a role in stabilizing activated eNOS in the non-caveolar subcellular domain to further maintain increases in NO production.²⁸ This chaperon protein HSP90 is also found to co-immunoprecipitate with eNOS under basal unstimulated condition in bovine aortic endothelial cells²⁸ and within the caveolae microdomain in ovine uterine artery endothelial cells.²⁹ HSP90 and eNOS interaction was observed to increase after receptor-mediated activation of eNOS; which was confirmed by the use of HSP90 specific inhibitor, Geldanamycin that leads to a reduced NO production.²⁸

eNOS PROTEIN-PROTEIN INTERACTION

eNOS activity is further regulated by its interaction with specific endothelial proteins including cav-1, CaM, HSP90, eNOS Interacting Protein (NOSIP), eNOS traffic inducer (NOSTRIN) and others. We have reviewed the negative regulatory effects cav-1 has on eNOS activation, thus our focus will be on other regulatory proteins. CaM is an important

protein that positively regulates eNOS activity state due to its sensitivity to rises in intracellular Ca^{2+} . Early studies identified the specific eNOS amino acid residues that are important for calmodulin binding to be Phe-498, Lys-499, and Leu-511 in the bovine sequence.³⁰ The α -helical eNOS binds, via hydrophobic interactions, in a classical antiparallel orientation with respect to the terminal lobes of CaM.³¹ These studies further demonstrated that the inhibitory eNOS-cav-1 interaction is disrupted by binding of CaM to eNOS, leading to enzyme activation.³² Moreover, CaM binding completely reversed the eNOS enzyme inhibition induced by a synthetic oligopeptide, corresponding to the cav-1 scaffolding domain.³³ CaM binding also facilitated the association of eNOS subunits although it was suggested that this dimerization alone may not be sufficient for activation of the enzyme.³⁴ Specifically CaM completely reversed cav-1-induced inhibition of eNOS by enhancing the reductase domain electron transfer,³⁵ whereas at high intracellular Ca^{2+} concentrations, stimulation of eNOS reductase activity was observed without further changes in CaM binding.³⁶ CaM activation of eNOS also makes the enzyme more prone to depalmitoylation mediated by acyl-protein thioesterase, a step needed for eNOS translocation.³⁷ Furthermore, immunoprecipitation of cav-1 from bovine lung microvascular endothelial cells showed recovery of not only eNOS, but also HSP90 while further demonstrating that HSP90 facilitates CaM-induced dissociation from the eNOS-cav-1 complex.³⁸ In Figure 2 and 3B we illustrate the increased protein-protein interaction of eNOS and HSP90 as well as the decreased interaction of eNOS and cav-1. Fluid shear stress and agonists like VEGF or histamine activate eNOS via increased association with HSP90.³⁹ Estradiol-17 β also induced the interaction of HSP90 with eNOS and binding was abolished by ICI 182,780 (non-subtype specific estrogen receptor antagonist).⁴⁰ Heat shock in rats resulted in a significant increase in eNOS, HSP90, and HSP70 protein levels in the aorta.⁴¹ Furthermore, HSP90 and active AKT together increased eNOS activity by ~9 fold, which was abolished by disrupting HSP90-eNOS binding using Geldanamycin.⁴² Statins which are 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors promote tyrosine phosphorylation of HSP90 and the direct interaction of the M region of HSP90 with AKT and eNOS and finally the excitatory ^{ser1177}eNOS.^{43, 44} Furthermore, HSP90 binds to eNOS and triggers its transition from the Ca^{2+} -dependent to the phosphorylation-dependent states though this study examined ^{ser1177}eNOS only.⁴⁵

NOSIP is another protein that has been shown to bind to the carboxyl-terminal region of the eNOS oxygenase domain⁴⁶ and was reported to decrease NO synthesis by uncoupling the enzyme from the plasma membrane.⁴⁶ NOSTRIN was also demonstrated to bind to the eNOS C-terminal oxygenase domain (433–506) and was demonstrated to target the enzyme to vesicle-like structures with a concomitant drastic decrease in NO release.⁴⁷ In addition, several proteins are known to interact with eNOS including F- and G- actin,⁴⁸ vimentin,⁴⁸ PECAM-1,⁴⁹ etc.; however, they are beyond the scope of this review. Therefore, eNOS regulation involves multiple proteins that systematically coordinate the subcellular localization and the activity state of the enzyme.

eNOS MULTI-SITE PHOSPHORYLATION

Endothelial cell eNOS activation is modulated by post-translational modification via multi-site phosphorylation/dephosphorylation as illustrated in the basal vs. the stimulated state of

eNOS in Figure 2.^{50, 51} However, the sequential modifications of the multi-site phosphorylation state of eNOS within various subcellular domains are largely unknown. In Figure 3, we depicted a theoretical model for eNOS multi-site phosphorylation in the caveolar and non-caveolar subcellular domains of ovine uterine artery endothelial cells. The enzyme eNOS possesses multiple phosphorylation sites including ^{ser1177}eNOS and ^{ser635}eNOS, which are stimulatory, ^{thr495}eNOS, which is inhibitory, and ^{ser116}eNOS, which is stimulatory or inhibitory depending upon the agonist employed.^{17, 51, 52} Regulatory translocation events occur with activation of eNOS and increases in NO production. In Figure 3 we illustrate how shear stress or agonists (e.g. ATP, Estrogen, Bradykinin, Acetylcholine, VEGF and 8-Br-cAMP) signaling events that stimulate phosphorylation of ^{ser1177}eNOS and ^{ser635}eNOS and removal of ^{thr495}eNOS; which occurs concurrent with the translocation and the removal of tonic inhibition with cav-1. Although several excellent review articles^{2, 3, 19, 50, 53} have theorized the complex interaction between eNOS subcellular localization and its phosphorylation/dephosphorylation status, few studies have been performed to directly test this hypothesis.

Serine (ser) 1177

^{ser1177}eNOS is identified as the location of residue 1177 or 1179 and independent of species differences. ^{ser1177}eNOS is a widely studied excitatory phosphorylation site in the reductase domain near the C-terminus.¹⁷ Stimulation of ^{ser1177}eNOS phosphorylation via mutation of ser to aspartate showed enhanced enzyme activity as measured using the arginine to citrulline assay.⁵⁴ In contrast, prevention of ^{ser1177}eNOS phosphorylation via mutation of ser with alanine resulted in activity levels similar to that of controls.⁵⁴ Several calcium mobilizing agonists increase levels of endothelial ^{ser1177}eNOS phosphorylation: Bradykinin transiently stimulated phosphorylation of ^{ser1177}eNOS in BAEC,⁵⁵ porcine aortic endothelial cell, and human umbilical vein endothelial cells (HUVEC)⁵⁶ and these effects were blocked completely by wortmannin and LY294002 (inhibitors of phosphatidylinositol 3-kinase)⁵⁵ or the CaM-dependent kinase II (CaMKII) inhibitor KN-93.⁵⁶ Adiponectin significantly increased production of BAEC NO by ~3-fold accompanied by ^{ser1177}eNOS phosphorylation that was able to be inhibited by wortmannin, but was still phosphatidylinositol 3-kinase/AKT-independent and partially dependent on AMPK.⁵⁷ Epidermal growth factor (EGF), histamine, and thrombin also lead to increased HUVEC ^{ser1177}eNOS levels.⁵⁸ Furthermore, wortmannin completely blocked EGF-mediated ^{ser1177}eNOS phosphorylation, whereas histamine- and thrombin-mediated phosphorylation was Ca²⁺ dependent, without the involvement of PKC, or CaMKII, but instead worked in an AMPK mediated manner, independent of P13K-AKT.⁵⁹ Vascular endothelial growth factor (VEGF) effects on ^{ser1177}eNOS phosphorylation can be noted from a study that utilized phospho-mimetic and non-phosphorylatable eNOS constructs and demonstrated VEGF mediated endothelial cell migration via ^{ser1177}eNOS phosphorylation.⁶⁰ In BAECs, Adenosine-3',5'-triphosphate (ATP) was also demonstrated to phosphorylate BAEC ^{ser1177}eNOS.⁶¹ Shear stress-dependent phosphorylation of ^{ser1177}eNOS is PKA-dependent mediated mechanism not an AKT-dependent mechanism.⁶² Acetylcholine is also reported to lead to phosphorylation at ^{ser1177}eNOS.⁶³ Thus, a variety of agonists result in increased levels of the excitatory ^{ser1177}eNOS via specific second messenger systems.

Serine (ser) 635

ser⁶³⁵eNOS is identified as the location of residue 633 or 635 and independent of species differences. ser⁶³⁵eNOS is a well known excitatory phosphorylation site located in the CaM autoinhibitory domain of eNOS.¹⁷ Using a series of synthetic polypeptide fragments, significant inhibition of enzyme activity was observed with eNOS 601–633(635) in the FMN binding domain.⁶⁴ Phospho-peptide analysis utilizing MALDI-based mass spectrometry showed a two fold increase in purified eNOS protein activity (rate of conversion of L-[14C] arginine to L-[14C] citrulline).^{65, 66} The phospho-mimetic mutation of ser to aspartate at ser 635 also confirmed phosphorylation of ser⁶³⁵eNOS.^{65, 66} Similar to ser¹¹⁷⁷eNOS, shear stress, VEGF, and 8-Br-cAMP induced phosphorylation of ser⁶³⁵eNOS.¹² However, shear stress-stimulated ser⁶³⁵eNOS phosphorylation was not affected by phosphoinositide-3-kinase inhibitors wortmannin and LY-294002, but was blocked by PKA inhibitor H89 or infecting them with a recombinant adenovirus-expressing PKA inhibitor¹². Furthermore, maximum NO production from S635D-expressing cells was significantly higher than that of both wild type and S635A in both basal and increased [Ca²⁺]_i states showing that ser⁶³⁵eNOS phosphorylation is Ca²⁺-independent.⁶⁷ Furthermore, the MEK inhibitor U0126 decreased ATP-stimulated eNOS activity in COS-7 cells and decreased ser⁶³⁵eNOS levels.⁶⁸ In UAECs, ATP stimulation leads to an increase in ser⁶³⁵eNOS in both caveolar and non-caveolar domains.^{23, 51} Although much information regarding subcellular domain-specific alterations of this site is lacking, it has been reported that phosphorylation on ser⁶³⁵eNOS peaks between 5 and 10 min with sustained levels up to 30 min²³ and that this site is important for sustained NO production.^{17, 67}

Threonine (thr) 495

thr⁴⁹⁵eNOS is identified as the location of residue 495 or 497 and independent of species differences. thr⁴⁹⁵eNOS is an inhibitory phosphorylation site that is located in the CaM binding domain of the enzyme.⁶⁹ In an early study, Matsubara et al. synthesized a 20-amino acid peptide corresponding to the putative CaM binding domain of eNOS that formed a stoichiometric complex with CaM and demonstrated that this synthetic peptide was phosphorylated *in vitro* by protein kinase C.⁷⁰ Subsequently, another study showed that thr⁴⁹⁵eNOS is constitutively phosphorylated in porcine aortic endothelial cells and is quickly dephosphorylated following bradykinin treatment.⁵⁶ thr⁴⁹⁵eNOS dephosphorylation was entirely Ca²⁺-dependent and was inhibited by the PP1 phosphatase inhibitor calyculin A.⁵⁶ PKC was then shown to regulate eNOS activity by changing the binding of calmodulin.^{56, 70, 71} The partially specific PKC inhibitor Ro-318220 inhibited thr⁴⁹⁵eNOS phosphorylation whereas the inactive Ro-310645 isomer did not exhibit a such an effect.⁷¹ Mutation of thr 495 to alanine enhanced calmodulin binding to eNOS in the absence of agonists, whereas the corresponding non-phosphorylatable eNOS construct was not able to bind to CaM.⁵⁶ The phosphatase PP1 was then demonstrated to be involved in the dephosphorylation of thr⁴⁹⁵eNOS based on its specificity for this site whereas PP2A was implicated in the dephosphorylation of ser 1177.⁷¹ Furthermore, coordination among phosphorylation sites has been reported; signaling events altered phosphorylation at both ser 1177 and thr 495 site.⁷² In UAECs, the inhibitory thr⁴⁹⁵eNOS was mainly detected in the caveolar subcellular domain as illustrated in Figure 3.⁵¹ Thus, the PKC-

dependent ^{thr495}eNOS phosphorylation inhibits eNOS-calmodulin interaction and subsequent re-partitioning of the enzyme into the non-caveolar domain and its dephosphorylation by Ca²⁺ mobilizing agonists contributing to elevated eNOS activity.^{17, 23}

Serine (ser) 116

Ser 116 is identified as the location of residue 114 or 116 and independent of species differences. ^{ser116}eNOS is an agonist-dependent phosphorylation site that can be excitatory or inhibitory. This is the only site identified in the oxygenase domain of the enzyme.¹⁷ VEGF promotes dephosphorylation of ^{ser116}eNOS in cultured endothelial cells.⁷³ In contrast, other studies have shown significant increases in phosphorylation of ^{ser116}eNOS in response to HDL and ApoA1.⁷⁴ In BAECs, ATP (10 μM) resulted in delayed dephosphorylation of ^{ser116}eNOS,⁶¹ whereas in UAECs, ATP potently phosphorylated ^{ser116}eNOS.⁵¹ Additionally in UAECs, the increase in ^{ser116}eNOS was observed in the non-caveolar domain (Figure 3). In one study, a phosphorylation-deficient mutant of eNOS in which ser 116 was mutated to alanine demonstrated enhanced enzyme activity compared with the wild-type.⁷³ In contrast, another study showed that mutation of ser 116 to aspartate had no effect on basal NO production, whereas increased NO production was observed when stimulated compared with the wild type enzyme.⁶¹

CONCLUSIONS

Nitric oxide production by endothelial cells under various physiologic states involves a series of complex regulatory processes that alter the activity of endothelial eNOS. These multifaceted processes require specific sub-cellular eNOS partitioning between plasma membrane caveolar domains and non-caveolar compartments. Therefore in normal healthy endothelial cells, eNOS activation and elevations in NO biosynthesis integrate eNOS translocation from the plasma membrane with its movement into the cell with alterations in the cellular milieu including its association with Ca²⁺. We integrated and presented data in this review interpreting information that demonstrated: 1) eNOS trafficking to caveolar receptor system; 2) eNOS trafficking and specific protein-protein interaction for its inactivation and activation; and 3) how these complex mechanisms relate to eNOS multi-site phosphorylation and signaling. Thus, this review may provide putative therapeutic clues that help understand several regulatory mechanisms that increase NO bioavailability and improve endothelial function. However, research is still needed to define the relative contribution of eNOS phosphorylation as it relates to preeclampsia and gestational diabetes and their effects on NO bioavailability. These endothelial dysfunctions, therefore, have life-long deleterious health consequences predisposing the offspring from these pregnancies to hypertensive disease, type II diabetes, adiposity, and other diseases as described by the Barker hypothesis.¹

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Fig. 1.

Transmission electron micrograph (TEM) identifying distinct caveolae structures in the uterine artery endothelial cell (UAEC) plasma membrane. Arrows point to the Ω -shaped caveolae invaginations. The UAEC were grown on coverslips and were fixed in a solution of 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4. The UAECs were post-fixed in 1% osmium tetroxide followed by OsO₄ postfixation. Samples were dehydrated in a graded ethanol series, then further dehydrated in propylene oxide and embedded in Spurr's epoxy resin. Samples were sectioned parallel or perpendicular to the coverslip surface for TEM using a Reichert-Jung Ultracut-E Ultramicrotome (Reichert, Inc., Depew, NY, USA) and contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% ethanol. Ultrathin sections were observed with a Philips CM120 electron microscope (Philips, Eindhoven, the Netherlands) and images were captured with a Mega View II side mounted digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Fig. 2.

Schematic representation of the regulatory mechanisms of endothelial nitric oxide synthase (eNOS). The post-translational modifications, protein–protein interactions and signalling enzymes that regulate eNOS activation state are illustrated for both basal and stimulated states. The eNOS protein contains a reductase and an oxygenase domain, with a Ca²⁺-calmodulin (CaM) binding domain between them. The reductase domain also houses the nicotinamide adenine dinucleotide phosphate (NADPH) binding domain close to the C-terminus, flavin adenine dinucleotide (FAD) and the flavin mononucleotide (FMN) close to the CaM binding domain. Stimulatory phosphorylation of ser1177eNOS and ser365eNOS is shown, along with the phosphatases and kinases reported to alter these phosphorylation sites. These stimulatory phosphorylation events are denoted by a green circle. The inhibitory phosphorylation of thr495eNOS is shown along with the phosphatase and kinases reported to alter this phosphorylation site. This inhibitory phosphorylation event is denoted by a red circle. The phosphorylation of ser116eNOS is shown along with the phosphatase and kinases reported to alter this phosphorylation site. This phosphorylation event is denoted by a green circle and a red P to underscore the difference in its effect on Enos activation; which is agonist dependent. A list of common agonists reported to alter eNOS phosphorylation pattern and/or protein–protein interactions are also provided. VEGF, vascular endothelial growth factor; PP1, serine/threonine phosphoprotein phosphatase 1; PP2/PP2A, serine/threonine protein phosphatase 2 or 2A; PP2B or Calcineurin, a protein phosphatase; PKC, PKA, protein kinase C and A, respectively; AMPK, AMP-activated protein kinase; BH₄, tetrahydrobiopterin; 8-Br-cAMP, 8-bromo-cAMP; EGF, epidermal growth factor; CaMKII, calmodulin kinase II; ERK, extracellular signalregulated kinase; HSP90, heat shock protein 90.

Fig. 3.

Theoretical regulatory translocation model for endothelial nitric oxide synthase (eNOS) activation in ovine uterine artery endothelial cells (UAEC). (a) A schematic representation of an unstimulated caveolae microdomain in which eNOS is shown with its basal phosphorylation state along with the tonic eNOS inhibition when bound to caveolin (cav)- 1. (b) Representation of shear stress or agonists (calcium-mobilizing agonists) and signalling events that stimulate phosphorylation of ser1177eNOS and ser635eNOS and the removal of thr495eNOS concomitant with its translocation and removal of tonic inhibition by the association of eNOS with cav-1. ER, endoplasmic reticulum. CaM, Ca²⁺ calmodulin; Hsp90, heat shock protein 90.21,23,50

Table 1

Functions of proteins identified and shown to localize in the Caveolae of endothelial cells

Protein	Name and Function	Reference
Membrane proteins		
PDGF-R	Platelet-derived growth factor receptor. Cell surface tyrosine kinase receptors; which regulate cell proliferation, cellular differentiation and cell growth.	75
CD36	Class B scavenger receptor family that mediates the endocytosis of multiple ligands such as long-chain fatty acids	76, 77
gp60	cell surface albumin-binding glycoprotein that regulates endothelial permeability of albumin	78
Flk-1/KDR	Cell surface tyrosine kinase also known as VEGF receptor. This kinase is associated angiogenesis	79, 80
TFPI	Tissue factor pathway inhibitor downregulates the procoagulant activity of tissue factor	81
Kv1.5 channels	Voltage-dependent potassium channel that restores resting membrane potential	82
MMP-X	Matrix metalloproteinase are capable of degrading many types of extracellular matrix proteins and are involve in tissue remodeling	29, 82, 83, 84
G protein-coupled receptors		
B2R	Bradykinin receptor which stimulates PLC to increase intracellular Ca^{2+} concentration	85
ET _A	Endothelin receptor activation increases intracellular Ca^{2+} concentration.	86,
AR α_1	Adrenergic receptors- α_1 associates with G _q heterotrimeric G-protein after epinephrine or norepinephrine binding to induce vasoconstriction in blood vessels	87
Guanine Nucleotide-binding Protein (G proteins)		
G α_s	Regulates G protein-coupled receptor activity	76
G α_{i1}	Regulates G protein-coupled receptor activity	76
G α_{i2}	Regulates G protein-coupled receptor activity	76
G $\beta\gamma$	Regulates G protein-coupled receptor activity	76
Gq	Regulates G protein-coupled receptor activity	76, 88
Non-receptor tyrosine kinases		
c-Src	Regulation of growth factor response	76, 75, 87
Fyn	Regulation of growth factor response	76, 75, 87
Yes	Regulation of growth factor response	76, 75
Lck	Regulation of growth factor response	76, 75
Lyn	Regulation of growth factor response	76, 75, 87
Tyk2	Regulation of growth factor response	85
STAT3	Signal transducers and activators of transcription	85
Nonreceptor Ser/Thr kinases		
Raf	Signal transduction of mitogenic signals	89
MEK	Mitogen-activated protein kinase responsible for signal transduction of mitogenic signals	76
PI-3 kinase	Phosphatidylinositide 3-kinase phosphorylates phosphatidyl-inositol for signal transduction	76, 75
PKC α , β	Protein Kinase C is a protein Ser/Thr kinase	76, 75
Other enzymes		

Protein	Name and Function	Reference
eNOS	Endothelial nitric oxide synthase catalyzes the production of nitric oxide	18, 25, 29
PLC γ	Phospholipase C- γ is a ubiquitous protein responsible for transmitting intracellular signals induced by hormones.	75
PTGIS/CYP8A1	Prostacyclin synthase is responsible for the production of prostacyclin (PGI ₂) that functions as a potent vasodilator inhibitor of platelet aggregation	90, 29
GTPases		
Ras	Small GTPase responsible for signaling transduction at the intracellular region of transmembrane receptors	76, 29
Rap1	Ras-related protein-1 is a small GTPase responsible for signaling transduction at the intracellular region of transmembrane receptor	76
Rap2	Ras-related protein-1 is a small GTPase responsible for signaling transduction at the intracellular region of transmembrane receptors	76
Cellular proteins/adaptors		
Shc	An adaptor protein that regulates growth factor response	76
Grb2	Growth factor receptor-bound protein 2 responsible for signaling transduction through its SH2 and SH3 domains	76, 91
Gab1	Grb2-associated binding protein 1 is a docking protein that mediates interactions between receptor tyrosine kinases and non-receptor tyrosine kinases for cell signaling	91
Other proteins		
ER α and β	Estrogen receptors α and β are hormone receptors that bind estradiol-17 β	92, 25, 93, 94
NCX	Na ⁺ /Ca ²⁺ exchanger	95
HspX	Heat shock proteins: 60, 70, 78, or 90 known as chaperon to help maintain proteins folding and stability	29, 28
AR	Androgen receptor	92
FGFR1	Fibroblast growth factor receptor-1 responsible for cellular mitogenesis and differentiation	91

Proteins that have been found to localize within the caveolae of endothelial cells. The methods of detection vary among studies; however, results yield similar conclusions. Caveolae were show to comprise of a significant number of cell signaling components, from receptors to adaptor proteins, to kinases and their targets/effectors proteins. All the protein interactions within the caveolar domain facilitate both regulatory mechanisms and signaling propagation.

Table 2

Kinases and Phosphatases that regulate eNOS Activity

Phosphorylation site	Kinase/*Phosphatase	eNOS Activity Level
Threonine 495	*PP1 and *PP2B	Up
	PKC and AMP-K	Down
Serine 1177	Ras->PI3-K->PDK->Akt	Up
	Ras/Raf->MEK->ERK	Up
	Gα/Gβ->cAMP->PKA	Up
	CaMKII	Up
	AMP-K	Up
	*PP2A	Down
Serine 635	Ras/Raf->MEK->ERK	Up
	PKA or PKG	Up
Serine 116	Ras->PI3-K->PDK->Akt	Up
	PKC	Up or Down
	*PP2B	Down

Signaling cascades that have been identified to be responsible for increases or decreases in eNOS activity levels. Phosphorylation is one level of eNOS regulation. Other regulatory mechanisms include protein-protein interactions and changes in intracellular Ca^{2+} .