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Regulation of Endothelial Nitric oxide Synthase Activity by Protein-Protein Interaction

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Abstract

Endothelial nitric oxide synthase (eNOS) is expressed in vascular endothelial cells and plays an important role in the regulation of vascular tone, platelet aggregation and angiogenesis. Protein-protein interactions represent an important posttranslational mechanism for eNOS regulation. eNOS has been shown to interact with a variety of regulatory and structural proteins which provide fine tune-up of eNOS activity and eNOS protein trafficking between plasma membrane and intracellular membranes in a number of physiological and pathophysiological processes. eNOS interacts with calmodulin, heat shock protein 90 (Hsp90), dynamin-2, β -actin, tubulin, porin, high-density lipoprotein (HDL) and apolipoprotein AI (ApoAI), resulting in increases in eNOS activity. The negative eNOS interacting proteins include caveolin, G protein-coupled receptors (GPCR), nitric oxide synthase-interacting protein (NOSIP), and nitric oxide synthase trafficking inducer (NOSTRIN). Dynamin-2, NOSIP, NOSTRIN, and cytoskeleton are also involved in eNOS trafficking in endothelial cells. In addition, eNOS associations with cationic amino acid transporter-1 (CAT-1), argininosuccinate synthase (ASS), argininosuccinate lyase (ASL), and soluble guanylate cyclase (sGC) facilitate directed delivery of substrate (L-arginine) to eNOS and optimizing NO production and NO action on its target. Regulation of eNOS by protein-protein interactions would provide potential targets for pharmacological interventions in NO-compromised cardiovascular diseases.

Introduction

Nitric oxide synthase (NOS) catalyzes the reaction that oxidizes L-arginine in the presence of molecular oxygen and NADPH and simultaneously produces NO and stoichiometric amounts of L-citrulline [1;2]. Nitric oxide (NO) is a biologically active gas that functions as a potent signaling molecule in a number of physiological and pathophysiological processes such as neuronal communication, host defense, the regulation of vascular tone, platelet aggregation, and angiogenesis [2]. There are three different isoforms of NOS which can be divided into two general categories: constitutive and inducible. Constitutive NOS includes endothelial NOS (eNOS) and neuronal NOS (nNOS) that are regulated by calcium and calmodulin and produce NO in low concentrations for physiological purposes. Inducible NOS (iNOS) is calcium-insensitive due to its binding to calmodulin, and is mainly expressed

by inflammatory cells and produces NO in high concentrations which functions in host defense. eNOS is expressed in vascular endothelial cells and plays an important role in the regulation of vascular tone and angiogenesis. eNOS is also expressed in a variety of other cell types such as bronchiolar and kidney epithelial cells, cardiomyocytes and neutrophils [1]. eNOS is tightly regulated by transcriptional, posttranscriptional and posttranslational mechanisms [3;4]. eNOS has been shown to interact with a variety of regulatory and structural proteins (Table 1). Protein-protein interactions represent an important posttranslational mechanism for eNOS regulation [3]. This review will focus on the eNOS interacting proteins and their contributions to the physiological and pathophysiological processes associated with eNOS activity.

Calmodulin

eNOS activity depends on calcium concentration [1]. It has long been known that calmodulin serves as an allosteric activator for eNOS [5–7]. eNOS is activated when intracellular Ca^{2+} levels increase sufficiently to maintain calmodulin binding. The binding of eNOS to calmodulin is reversible, as calmodulin is released and eNOS becomes inactive when intracellular Ca^{2+} concentration decreases to basal level. The calmodulin binding domain of bovine eNOS is located at residues 493–512 (human residues 492–511) that form a basic and amphiphilic α -helix within the protein [7]. The affinity for calmodulin to bind eNOS is 10^{-9} M in the presence of Ca^{2+} [8]. Binding to calmodulin displaces adjacent autoinhibitory loop on eNOS and promotes electron transfer from oxygenase domain to reductase domain [5]. Calmodulin binding facilitates eNOS dimerization [9], which is important for eNOS activity *in vivo*. In addition, calmodulin binding simultaneously disrupts the inhibitory eNOS-caveolin complex leading to enzyme activation [10].

Phosphorylated calmodulin has less affinity to eNOS. Calmodulin phosphorylation by CK2 kinase leads to selective disassociation of calmodulin from eNOS regardless of intracellular Ca^{2+} levels [11]. A number of pathways converge on mobilization of intracellular calcium transients to cause the most rapid mechanisms of eNOS activation via calmodulin. G protein-dependent signaling pathway triggered by agonists such as bradykinin, acetylcholine, or estradiol provide an important pathway for intracellular Ca^{2+} elevation and eNOS activation [12].

Heat-shock protein 90 (Hsp90)

Hsp90 is known to serve as a molecular chaperone in protein folding and maturation events. Two genes encode Hsp90, with the human gene products Hsp90 α and Hsp90 β having 86% sequence homology. The Hsp90 is highly abundant in endothelial cells, accounting for 1 to 2% of cytosolic protein, and is localized to the cytoplasm, and a small amount of Hsp90 is found in the nucleus and cytoskeleton. Hsp90 also serves as an allosteric activator of eNOS [13]. ENAP-1 (endothelial nitric oxide synthase-associated protein 1), a protein initially identified as a partner to eNOS was later identified as Hsp90 [14;15]. An earlier experiment using a yeast two-hybrid system shows that hsp90 interacted with eNOS at amino acid (aa) 300–400 of eNOS [16]. A later experiment using site-directed mutagenesis pinpoints the Hsp90 binding site to be aa 310–323 in bovine eNOS, with Glu-310, –314, –318, and –323

for hsp90-eNOS interactions [17]. Hsp90 binds to eNOS through its middle M domain [16]. Hsp90 binding stimulates eNOS activity by cooperatively enhancing the affinity of eNOS for calmodulin, by balancing output of NO versus superoxide, and by facilitating heme binding [16–19]. More importantly, the charged middle M domain of Hsp90 contains independent binding sequences for both eNOS and Akt. Hsp90 binding increases the affinity of Akt for eNOS and/or unmask the phosphorylation sites on eNOS, which increases the rate of Akt-dependent eNOS phosphorylation [16;20].

Vascular endothelial growth factor (VEGF), histamine, estrogen, and fluid shear stress promote rapid association of Hsp90 with eNOS in endothelial cells and augment eNOS activity and NO release [13]. Blockade of Hsp90-mediated signalling limits both agonist-induced NO production and vasorelaxation [13;17]. Hsp90 association with eNOS is compromised in a number of pathological processes. Hypoxia reduces eNOS interaction with Hsp90 and decreases eNOS activity in pulmonary hypertension [21–23]. Hyperglycemic conditions reduces Hsp90-eNOS complex formation and NO production *in vivo* in rat mesenteric arteries [24]. Both hypoxia- and hyperglycemia-induced reduction of eNOS-Hsp90 association appear to be mediated by calpain, a calcium-dependent protease [21;24]. The inhibition of eNOS activity by C-reactive protein at inflammatory condition is contributed to decreased binding of eNOS to Hsp90 [25]. The therapeutic effect of statins is related to tyrosine phosphorylation of hsp90, which facilitates the ability of hsp90 to bind and activates Akt and eNOS [26]. PPAR γ ligands including the endogenous 15-deoxy-12,14-prostaglandin J2 (15d-PGJ2) and some thiazolidinediones like rosiglitazone act in part by promoting the interaction of hsp90 and eNOS, resulting in Ser 1177 phosphorylation [27]. Metformin, a drug for the treatment of type 2 diabetes also dramatically attenuates high glucose-induced reduction in the association of Hsp90 with eNOS in bovine aortic endothelium, resulting in increased NO release [28]. Furthermore, NO itself may cause S-nitrosylation of cysteine residues of Hsp90 and inhibit eNOS-Hsp90 interaction, which functions as negatively feedback mechanism for eNOS regulation [29].

Dynamin-2

Dynamin-2 is a large ubiquitously expressed GTP-binding protein that is targeted to Golgi membranes and colocalizes with caveolin within caveolae [30;31]. It functions in vesicle formation, receptor-mediated endocytosis, caveolae internalization and vesicle trafficking in and out of the Golgi [32]. Dynamin-2 also modulates signaling pathways by the means of distinct protein interactions [31]. Dynamin-2 has been shown by confocal microscopy to colocalize with eNOS in the Golgi membranes of endothelial cells and to bind eNOS directly, both *in vitro* and *in vivo* [33]. In *in vitro* assays, dynamin-2 directly increases NOS activity and in cells, the association between eNOS and dynamin-2 is boosted by calcium ionophore [33]. Dynamin-2 binds the eNOS reductase domain and increases eNOS activity by potentiating electron transfer [34]. It has been shown that dominant-negative mutants of dynamin-2 and caveolin prevents caveolae-mediated eNOS internalization and endothelial hyperpermeability induced by agonists such as platelet-activating factor and VEGF [35;36]. Coordinating with caveolin, nitric oxide synthase-interacting protein (NOSIP), nitric oxide synthase trafficking inducer (NOSTRIN) and cytoskeleton, dynamin-2-eNOS interaction

may play a role in eNOS trafficking between plasma membrane and intracellular membranes [37;38].

Cytoskeletal proteins

eNOS is associated with the actin cytoskeleton, microtubules, and intermediate filaments in a direct or indirect manner [15;39–42].

There is a significant amount of eNOS in the insoluble portion of the triton-extraction of aortic endothelial cells [15]. The triton-insoluble fraction represents mainly the actin microfilaments. eNOS localized to the plasma membrane is colocalized with cortical F-actin. eNOS that is located in the perinuclear area (probably Golgi) is colocalized with G-actin [41]. Immunoprecipitation of eNOS from the caveolar fraction of PAEC plasma membranes resulted in the co-precipitation of β -actin, and immunoprecipitation of actin from the caveolar fraction of pulmonary artery endothelial cell plasma membranes resulted in the coprecipitation of eNOS, suggesting that eNOS is associated with β -actin protein [41]. A yeast-two hybrid experiment confirmed that the eNOS oxygenase domain rather than the reductase domain or the middle part of the eNOS molecule has direct interaction with β -actin, suggesting that the actin-binding site is in the oxygenase domain of the eNOS protein [43]. The actin binding site in eNOS might be at residues 326–333 of the eNOS oxygenase domain [44]. Hydrophobic residues leucine-326, leucine-328, tryptophan-330, and leucine-333 in residues 326–333 of eNOS oxygenase domain are essential for β -actin binding [44].

The association of eNOS with the actin cytoskeleton is also indirect through other eNOS interacting proteins. Among the eNOS-interacting proteins, caveolin, calmodulin, Hsp90, dynamin-2, CAT-1, NOSTRIN and NOSIP have been reported to connect to actin cytoskeletal proteins [45–51]. eNOS-actin can form ternary complex with these proteins. For example, Hsp90 forms a tight complex with NOS-3 and G-actin in platelets and this binding gives rise to an increase in the rate of Hsp90 degradation on the ternary complex *in vivo*. This may serve to provide a negative-feedback mechanism, thereby limiting the time for which NOS-3 is in the activated state, such that, once Hsp90 is degraded, NOS-3 is released from G-actin [52].

Binding of G-actin to eNOS increases its activity *in vitro* [41;44;53]. The increase in NO production induced by β -actin binding to eNOS is accompanied by decrease in superoxide production, suggesting that β -actin binding to eNOS shifts the enzymatic activity from superoxide formation toward NO production [44]. In addition, association of G-actin with eNOS plays an essential and necessary role in agonist-induced eNOS activation, through enabling its phosphorylation by Akt at serine residue 1177 [53].

Incubation of purified eNOS with either F-actin or G-actin results in significant increases in eNOS activity [41]. However, incubation of eNOS with G-actin results in a more significant increase in eNOS activity than that observed with F-actin. Moreover, the magnitude of increase in eNOS activity caused by swinholide A-induced disruption of F-actin is much greater than that in eNOS protein content. Stabilization of F-actin by phalloidin decreases

eNOS activity without affecting eNOS protein content. These results indicate that the ratio of G-actin to F-actin regulates eNOS activity [41]. Lower eNOS activity while associated with polymerized F-actin may help prevent undesired activation of eNOS in the trafficking of eNOS from the plasma membrane to intracellular cytoskeletal structures induced by NOSTRIN and NOSIP [51;54].

Besides its role in eNOS trafficking, alterations of eNOS-actin interaction have been found to be involved in several physiological and pathophysiological events. First, association of eNOS with G-actin plays an important role in agonist-induced eNOS activation [53]. It has been demonstrated that eNOS agonists adenosine, salbutamol, histamine and thrombin all increase association of eNOS with G-actin [53]. Stabilization of filamentous actin using phalloidin inhibits the increase of eNOS/ β -actin association, Ser1177 phosphorylation, eNOS activity and cGMP production. Second, endothelial growth induces dynamic alteration in eNOS activity and NO production due to changes in the interaction of eNOS to cytoskeleton. Quiescent pulmonary artery endothelial cells (100% confluent) have increased association of G-actin and F-actin to eNOS, comparing to more rapidly-replicating pre-confluent cells [43]. This finding provides a solid explanation for the observation that eNOS enzymatic activity in pulmonary artery endothelial cells is six-fold greater in quiescent cells than in pre-confluent cells [55]. Third, hypoxia decreases the association of eNOS with F-actin and G-actin [41], which might be responsible for decreased eNOS activity and NO production during hypoxia. Fourth, increased association of eNOS with actin in pulmonary vascular endothelial cells contributes to hyperoxia-induced increase in the production of peroxynitrite which may cause nitrosative stress in pulmonary vasculature [56]. Manipulation of eNOS-actin association may render novel therapeutic method to treat diseases associated to hyperoxic injuries.

eNOS is also associated with microtubules. eNOS protein can be coimmunoprecipitated by anti-tubulin antibody [40]. Modifications of tubulin polymerization by either taxol or nocodazole do not influence eNOS-tubulin association but do affect eNOS-Hsp90 interactions. Pharmacological stabilization of microtubules increases association of eNOS with Hsp90, eNOS activity, and NO production. Disruption of microtubules decreases association of eNOS with Hsp90, eNOS activity, and NO production [40]. Association of eNOS with vimentin has been confirmed in sertoli cell tight junctions [42]. However, the effects on eNOS activity and the binding site have not been clarified.

Porin

Porin, a 35-kDa voltage-dependent anion channel (VDAC) has been identified to be an eNOS associated protein [57]. The interaction of eNOS and porin in plasmalemmal caveolae is direct and specific, enhanced by stimulators of eNOS, and ultimately leads to increased eNOS activity [57]. Treatment of the human endothelial cells with either a calcium inophore or bradykinin markedly enhanced the association of porin with eNOS. Porin contains multiple calcium-binding sites, which allows it to participate in intracellular calcium signaling and transport. The binding of calcium to porin may produce a favorable conformational change that allows it to bind efficiently to eNOS. In persistent pulmonary hypertension of the newborn (PPHN), porin protein is decreased [58]. The ability of agonists

to increase the eNOS/VDAC interaction was significantly blunted in pulmonary artery endothelial cells from fetal lambs with chronic intrauterine pulmonary hypertension, contributing to the limited eNOS activity in PPHN [58].

High-density lipoprotein (HDL) and apolipoprotein AI (ApoAI)

HDL and ApoAI, a major apolipoprotein of HDL, induces up-regulation of eNOS activity via protein-protein interaction of eNOS and ApoAI [59]. Cross-linking, co-immunoprecipitation, and co-localization studies show that ApoAI is associated with eNOS in endothelial cells [59]. HDL and ApoAI may bind to a cell surface receptor (SR-BI or other HDL receptor) and triggers endocytosis response. The ApoAI then moves to the Golgi and binds eNOS. During this process the necessary initiating enzymes, such as AMP-activated protein kinase, are actively recruited and eNOS is then phosphorylated and activated [59].

Caveolin

Caveolins are 22 kDa proteins integrally linked to caveolae, which are specialized invaginations of the plasma membrane composed of cholesterol, glycosphingolipids, and other structural and signaling proteins including G-proteins and their coupled receptors [60]. Caveolin-1 and caveolin-2 are ubiquitously expressed and abundant in endothelial cells; caveolin-3 is a muscle-specific isoform expressed in cardiomyocytes and skeletal muscle [61]. eNOS could directly interact with caveolin-1 or caveolin-3 [62–65]. Caveolin over-expression in COS-7 cells resulted in a reduction of eNOS activity and a reduction in NO release [10;66]. The caveolin binding motif of eNOS is located within amino acids 350–358. Mutagenesis of this region within eNOS blocked the ability of caveolin to suppress NO release [66]. The primary binding region of caveolin-1 for eNOS is within amino acids 60–101 and, to a lesser extent, amino acids 135–178 [64;66]. The caveolin-eNOS immunocomplex is disrupted in the presence of caveolin scaffolding peptides (amino acids 82–101) [10]. Incubation of pure eNOS with peptides derived from the scaffolding domains of caveolin-1 and –3 results in inhibition of eNOS activity, further supporting that caveolin binding to eNOS inhibits its activity [66].

The inhibitory effect of caveolin on eNOS is achieved through blocking the calmodulin binding site in eNOS [10]. The reduction of eNOS activity by caveolin peptides, or over-expressed caveolin, is reversed by exogenous addition of calmodulin [10]. The interaction of calmodulin and/or caveolin-1 with eNOS is mutually exclusive [67]. Compartmentalization of eNOS in caveolae is necessary for reciprocal regulation of eNOS by calmodulin and caveolin in living endothelial cells. In the resting state, eNOS is tethered to caveolin-1 in caveolae, due to which the enzyme activity is repressed. After provocation by G-protein coupled receptor agonists that raise intracellular Ca^{2+} concentrations, such as bradykinin, calmodulin binds and caveolin dissociates from eNOS. When the tide of intracellular Ca^{2+} subsides, the cycle is reversed with calmodulin dissociating and caveolin reassociating with now-inactive eNOS [62].

G protein-coupled receptors (GPCR)

eNOS interactions with membrane-docking proteins are not restricted to interactions with the integral membrane protein caveolin-1 but also occur with the transmembrane GPCRs such as bradykinin B2 receptor, the Ang II AT1 and the ET-1 ETB receptors. eNOS can be co-immunoprecipitated with bradykinin B2 receptor, the Ang II AT1 and the ET-1 ETB receptors [68;69]. eNOS interactions with the receptors are direct and inhibitory and are mediated by a membraneproximal subdomain of the B2 receptor intracellular domain 4 [69]. Agonist binding to a GPCR triggers the activation of heterotrimeric G-proteins that transduce the signals to eNOS protein. Downstream of GPCR and heterotrimeric G proteins, two eNOS-activating mechanisms have been elucidated: mobilization of intra-cellular calcium and the phosphoinositide-3-kinase (PI3K)/Akt cascade. The GPCR pathways initiated by agonists such as bradykinin (B2 receptor), acetylcholine (m2 muscarinic receptor), histamine, adenosine, ADP/ATP, and sphingosine 1-phosphate (S1P), and the protein thrombin are coupled to G α q or G α i proteins that result in activation of phospholipase C and that mobilize intracellular calcium. The binding of Ca²⁺-calmodulin to eNOS disrupts the inhibitory eNOS-caveolin or eNOS-bradykinin B2 complex, leading to eNOS activation. GPCR is also coupled to downstream kinases like PI3K/Akt, which induces eNOS phosphorylation at Ser 1177 and eNOS activation [68].

NOSIP and NOSTRIN

NOSIP is a 34 kDa protein identified to interact with eNOS using yeast-two hybrid screening that binds the carboxy-terminal of the eNOS oxygenase domain [54]. NOSIP shares same binding site with caveolin on NOS since caveolin and NOSIP compete with one another to bind to the oxygenase domain of eNOS [54]. Overexpression of NOSIP diminished the NO output of eNOS, possibly by uncoupling eNOS from its caveolar attachments and disrupting interaction with caveolar co-localized effectors of upstream agonists [54]. NOSIP assists in translocation of eNOS from plasma membrane caveolae to the intra-cellular regions, with the actin cytoskeleton as a specifically identified destination [54]. Targeting to the cytoskeleton appears to be cell cycle dependent, occurring during G2 phase; cell cycle specific NOSIP-dependent eNOS targeting may be due to its tightly controlled nucleocytoplasmic shuttling, where nuclear export outweighs constitutive nuclear import only during G2, resulting in cytoplasmic accumulation of NOSIP during G2 phase [70].

NOSTRIN is another eNOS interacting protein identified using yeast-two hybrid screening with a molecular weight of 58 kDa [51]. NOSTRIN contains an N-terminal cdc15 domain consisting of an FCH region and a coiled-coil structure and C-terminal SH3 domains [71]. The FCH region is sufficient to direct NOSTRIN to membrane including plasmalemma and peripheral vesicles. The SH3 domain not only binds the oxygenase domain of eNOS, but also the GTPase dynamin and N-WASP (neural Wiskott–Aldrich syndrome protein) [38]. Caveolin-1 and NOSTRIN each enhance the binding of the other to eNOS on unique sites and help form a ternary eNOS–NOSTRIN–caveolin-1 complex [72]. NOSTRIN is the critical adaptor of a multimeric protein complex that binds and regulates dynamin-2 and N-WASP necessary for caveolar endocytosis and eNOS internalization [38]. NOSTRIN recruits

dynamin to caveolae and drives endocytosis by dynamin GTPase-mediated vesicle fission [38]. N-WASP and NOSTRIN co-localize with eNOS along the actin cytoskeleton. Because disruption of actin filaments traps NOSTRIN–eNOS at the peripheral membrane and perinuclear region, NOSTRIN–N WASP is thought to promote actin polymerization to help shuttle vesicular cargoes [38].

Overexpression of NOSTRIN triggers redistribution of eNOS from the plasma membrane to intracellular vesicular structures with a concomitant attenuation of the NO-producing capacity [51]. The inhibitory influence of NOSTRIN on eNOS, independent of translocation, might help prevent undesired activation of eNOS during its transit cycle [72].

NOSTRIN may be implicated in pre-eclampsia and portal hypertension associated with alcoholic hepatitis/cirrhosis [73;74]. It has been found that there are higher levels of NOSTRIN mRNA and protein in placenta of pre-eclampsia patients and livers of alcoholic hepatitis/cirrhosis patients. These are correlated with lower eNOS activity and NO release, suggesting NOSTRIN has a role in these conditions [73;74].

Cationic amino acid transporter-1 (CAT-1)

L-arginine, the substrate of eNOS, is transported across the plasma membrane by four different transport systems (y^+ , $b^{0,+}$, y^+L and $B^{0,+}$) [75;76]. The y^+ transport system is the main transporter that is responsible for 60–95% of carrier-mediated L-arginine delivery into lung vascular endothelial cells [77]. System y^+ transporter activity is attributed to a family of cationic amino acid transporters (CATs). The K_m of system y^+ (100–250 μM) lies within the physiological concentration range for circulating L-arginine. Four related CAT proteins have been identified and referred to as CAT-1, CAT-2 (A and B), CAT-3, and CAT-4 [78;79]. These CAT proteins constitute a subfamily of the solute carrier family 7 (SLC7). The gene names of SLC7A1, A2, A3, and A4 have been assigned to CAT-1, CAT-2, CAT-3, and CAT-4 respectively [78;79]. CAT-1 is the most extensively studied system y^+ protein. It was originally cloned and identified as the receptor for moloney murine leukemia virus (MMLV). Amino acid similarities observed between the MMLV receptor and L-histidine and L-arginine permeases from *Saccharomyces cerevisiae* led to the discovery of its physiological function as a Na^+ -independent CAT.

The rate of NO production in lung endothelium is critically dependent on the availability of L-arginine [80]. In physiological conditions, the concentration of circulating arginine is approximately 100 μM , and the tissue concentration ranges from 100 to 1000 μM . Several studies have shown that the K_m for eNOS is less than 10 μM . eNOS should be saturated in endothelial cells, and therefore increasing extracellular arginine should not increase NO production any further. However, a number of *in vitro* and *in vivo* studies indicate that NO production by vascular endothelial cells under physiological conditions can be increased by extracellular L-arginine despite a saturating intracellular L-arginine concentration [80;81]. This observation has been termed the “arginine paradox”. Attempting to understand this paradox, the existence of two arginine pools has been demonstrated in endothelial cells [82;83]. Pool-I can be depleted by extracellular lysine through an exchange mechanism mediated by membrane transporters such as the cationic amino acid transporter 1 (CAT-1).

Pool-II is not freely exchangeable with extracellular lysine, but accessible to eNOS, thereby rendering eNOS independent of extracellular arginine. Pool-II consists of recycled arginine from citrulline (Pool-IIA) and protein breakdown (Pool-IIB) [83].

A caveolar complex between CAT1 and eNOS has been found to exist in endothelial cells, which gives an evidence for the existence of two arginine pools and an explanation for the “arginine paradox” [84]. CAT-1 and eNOS proteins can be co-immunoprecipitated in the lysates of pulmonary endothelial cells. Immunohistochemical studies demonstrated that CAT-1, eNOS, and caveolin are co-localized in caveolae, suggesting that CAT directly delivers extracellular L-arginine to eNOS [84]. Pertussis toxin-induced activation of CAT-1 in pulmonary endothelial cells increased NO production without affecting eNOS activity [85], indicating that the caveolar CAT1-eNOS complex is interacting with L-arginine pool-I in endothelial cells. In addition, the actin-binding protein, fodrin, is linked to CAT-1, the major arginine transporter in the plasma membrane of endothelial cells [50]. Therefore, the eNOS-CAT-1-fodrin-actin complex within caveolae may provide a mechanism for the directed delivery of extracellular L-arginine to eNOS in pool-I. The existence of a CAT1-eNOS complex suggests that the K_m of the arginine transporter may be more important than the K_m of eNOS. The K_m of NO production by endothelial cells is approximately 73–150 μM [86], which is in the range of physiological L-arginine concentrations and the K_m values of the CAT-1 transporters [76].

Argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL)

Endothelial cells have an L-arginine biosynthetic pathway that converts L-citrulline to L-arginine [87]. This L-citrulline recycling pathway consists of a two-step enzymatic process [86]. In the first and rate-limiting step, L-citrulline is converted to argininosuccinate by argininosuccinate synthase (ASS) in the presence of L-aspartate and ATP. In the second step, argininosuccinate is converted to arginine by the action of argininosuccinate lyase (ASL). A gradient fractionation study has shown that ASS and ASL colocalize with eNOS in the caveolar fraction [88]. Further study indicates that inhibition of ASS reduces NO production in a dose-dependent manner, suggesting that the L-citrulline–L-arginine recycle is coupled to endothelial NO production [89]. It is not clear whether there is a direct association between ASS/ASL and eNOS. This L-citrulline–L-arginine recycle contributes to L-arginine pool-IIA which is closely associated with caveolae [83].

Soluble guanylate cyclase (sGC)

Soluble guanylate cyclase (sGC), a 72 kDa α/β -heterodimeric heme protein, catalyzes the conversion of GTP to cGMP [90]. Direct binding of NO to the sGC heme prosthetic group results in formation of the nitrosyl heme adduct and a conformational change in sGC that leads to activation of sGC. sGC activation in vascular smooth muscle cells produces vasorelaxation and inhibits smooth muscle proliferation. In vascular endothelial cells, the eNOS-sGC pathway is essential for VEGF-induced increases in EC permeability and proliferation. It has been found that a portion of total cellular sGC exists in a complex with eNOS and Hsp90 in vascular endothelial cells. In this sGC/eNOS/Hsp90 complex, eNOS-sGC interaction is indirect, with Hsp90 binding to both eNOS and sGC and functioning as

an adaptor protein for the eNOS-sGC interaction [91]. The function of sGC/eNOS/Hsp90 complex depends on Hsp90 being in an active conformation and is required for the action of bradykinin and VEGF on endothelial cells [91].

Summary

eNOS has been one of the proteins which has been extensively studied. It would not be surprising if more proteins are found to interact with eNOS in the coming years. Because eNOS is involved in many critical functions such as the regulation of vascular tone, platelet aggregation, and angiogenesis, eNOS interaction with other proteins allows fine tune-up of eNOS activity in these processes. In the mean time, regulation of eNOS by protein-protein interactions would provide potential targets for pharmacological interventions in NO-compromised cardiovascular diseases.

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Table 1.

Proteins that interact with endothelial nitric oxide synthase.

Protein	Effect of Bound Protein	References
Calmodulin	Activating	6,7,8,9,10
Hsp90	Activating	13, 14, 15, 16, 17, 18, 19, 20
Dynamin-2	Activating	33, 34
G-actin	Activating	41, 43, 44, 52, 53
Tubulin	Activating	40
Porin	Activating	57, 58
HDL and ApoAI	Activating	59
Calveolin	Inhibiting	62, 63, 64, 65, 66
GPCR	Inhibiting	68, 69
NOSIP	Inhibiting and trafficking	54, 70
NOSTRIN	Inhibiting and trafficking	38, 51, 72, 73, 74
F-actin	Trafficking	51, 54
CAT-1	Substrate delivering	84
ASS and ASL	Substrate delivering	88, 89
sGC	NO target	91

HDL (high-density lipoprotein); ApoAI (apolipoprotein AI), GPCR (G protein-coupled receptors); NOSIP (nitric oxide synthase-interacting protein); NOSTRIN (nitric oxide synthase trafficking inducer); CAT-1 (cationic amino acid transporter-1); ASS (argininosuccinate synthase); ASL (argininosuccinate lyase); sGC (soluble guanylate cyclase).