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invited review

Cellular regulation of endothelial nitric oxide synthase

ROLAND GOVERS AND TON J. RABELINK
Department of Vascular Medicine, University Medical Center Utrecht, Academic Hospital Utrecht, 3584 CX Utrecht, The Netherlands

Govers, Roland, and Ton J. Rabelink. Cellular regulation of endothelial nitric oxide synthase. *Am J Physiol Renal Physiol* 280: F193–F206, 2001.—Renal function is highly dependent on endothelium-derived nitric oxide (NO). Several renal disorders have been linked to impaired NO bioavailability. The enzyme that is responsible for the synthesis of NO within the renal endothelium is endothelial NO synthase (eNOS). eNOS-mediated NO generation is a highly regulated cellular event, which is induced by calcium-mobilizing agonists and fluid shear stress. eNOS activity is regulated at the transcriptional level but also by a variety of modifications, such as acylation and phosphorylation, by its cellular localization, and by protein-protein interactions. The present review focuses on the complex regulation of eNOS within the endothelial cell.

renal physiology; cardiovascular physiology; cell biology

ENDOTHELIUM-DERIVED NITRIC oxide (NO) has important effects on the renal vasculature. It modulates the constrictor actions on the afferent arteriole (35, 74, 75). In addition to its direct effects on glomerular microvascular tone, NO decreases the sensitivity of the tubuloglomerular feedback system (14). NO is also a crucial modulator of renal medullary blood flow, allowing pressure natriuresis (101, 135, 137). In addition, renal medullary oxygenation appears to be critically dependent on an intact vascular NO system (15). Finally, endothelium-derived NO prevents leukocyte infiltration of the vessel wall and thrombus formation in the renal vasculature (161). Related to these NO-dependent processes, a dysfunctional renal vascular NO system has been associated with (salt-sensitive) hypertension (9, 37), ischemic injury of the kidney (78, 82, 100), and tubulointerstitial renal disease (161, 162). It is of interest that these vascular effects of NO may not only be generated through the endothelial nitric oxide synthase (eNOS), which is the classic "vasculoprotective" NOS isoform but may also involve vascular expression of the neuronal (nNOS) and inducible isoforms (iNOS) of the enzyme (2, 81, 102). Nevertheless, functional studies suggest that the eNOS isoform is the key enzyme in these processes (102). New functions of eNOS in the kidney have recently been described as well. For example, the NO-mediated inhibition of chloride in the medullary thick ascending limb appears to depend on the endothelial isoform of NOS (124). The present review therefore focuses on the regulation of eNOS function. In particular, the structure-function relationships of the enzyme and its cellular regulation are reviewed.

FUNCTION AND ACTIVITY OF eNOS: NO VS. SUPEROXIDE

The free radical NO is generated exclusively by the enzyme NOS. Three isoforms of NOS have been identified, which are highly homologous in their primary structure (Fig. 1). Two isoforms are constitutively expressed, although their expression may be modulated: nNOS (or NOS1) is expressed in neurons, and eNOS (or NOS3) is expressed in endothelial cells, cardiac myocytes, and blood platelets. The expression of iNOS

Address for reprint requests and other correspondence: R. Govers, Dept. of Vascular Medicine, Univ. Medical Center Utrecht, Academic Hospital Utrecht-G02.228, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands (E-mail: R.M.T.Govers@LAB.AZU.NL).

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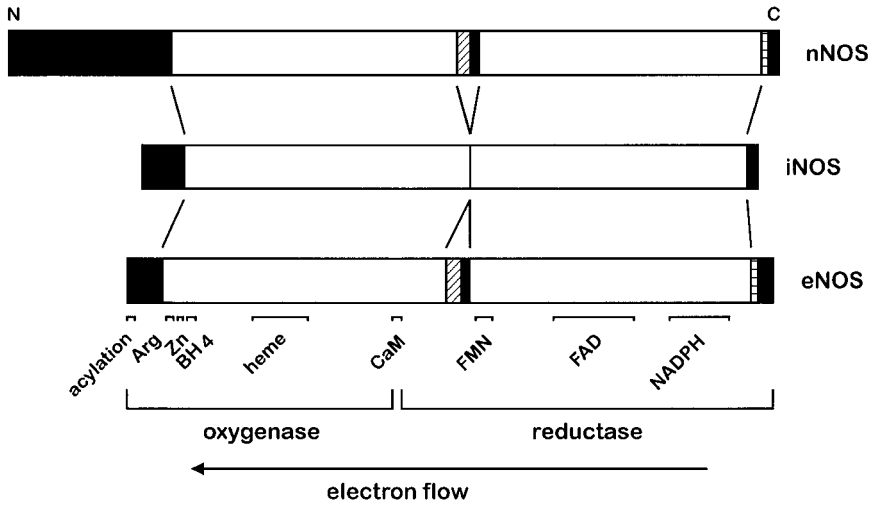

Fig. 1. Structure of nitric oxide isoforms. NH_2 (N) and COOH (C) termini are indicated. Homology in amino acid sequences are depicted: open boxes, homologous regions; hatched boxes, homologous in neuronal (nNOS) and endothelial nitric oxide synthase (eNOS); solid boxes, isoform-specific sequences. For eNOS, regions involved in acylation and in the binding of substrates and cofactors are indicated as well as the oxygenase and reductase domain and the direction of the intramolecular electron flow. Arg, arginine; BH4, tetrahydrobiopterin; CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

(or NOS2) is not constitutive but is induced by various cytokines. NO is synthesized from L-arginine through a five-electron oxidation step via the formation of the intermediate N^G-hydroxy-L-arginine (119, 171). The substrates for NOS-mediated NO production are the amino acid arginine, molecular oxygen, and NADPH. Cofactors that are required for NO generation are tetrahydrobiopterin (BH4), flavin adenine dinucleotide, and flavin mononucleotide. Furthermore, the enzyme contains binding sites for heme and calmodulin, both being essential for enzyme activity. After binding of calcium-loaded calmodulin to eNOS between the COOH-terminal reductase and NH_2-terminal oxygenase domain of eNOS, electrons are donated by NADPH at the reductase domain, which are subsequently shuttled through the calmodulin-binding domain toward the heme-containing eNOS oxygenase domain, which may result in the formation of the enzyme products citrulline and NO (1). eNOS also contains a motif involved in the binding of zinc (130). Each eNOS dimer contains one zinc ion, which plays a role in stabilization of the dimeric molecule.

Under certain conditions, NOS may generate superoxide instead of NO, a process called NOS uncoupling (i.e., uncoupling of NADPH oxidation and NO synthesis) (126). Superoxide generation by eNOS is mediated via the heme group of its oxygenase domain (147) and is dependent on the presence of its substrate, arginine, and its cofactor, BH4 (155, 165). When there is an abundance of both factors, eNOS produces NO. When the concentration of one of these factors is relatively low, eNOS generates superoxide. For instance, inhibition of BH4 synthesis in endothelial cells by 2,4-diamino-6-hydroxypyrimidine, an inhibitor of the rate-limiting enzyme in BH4 synthesis, GTP cyclohydrolase I, results in a reduction of NO synthesis, whereas eNOS-mediated superoxide synthesis is increased. This effect can be antagonized by the so-called BH4 salvage pathway, when endothelial cells are incubated with sepiapterin, which results in an increase in cellular BH4 levels, independent of GTP cyclohydrolase I (73). Similar findings have been reported for nNOS. The nNOS heme domain also produces superoxide when concentrations of arginine and/or BH4 are low (125, 154). In contrast, iNOS may also generate superoxide via its reductase domain (167). NOS uncoupling has also been reported in vivo. NOS-mediated superoxide generation has been suggested to occur in renal proximal tubules that were exposed to lipopolysaccharides (153), in renal arteries exposed to oxidized low-density lipoproteins (LDL) (129), in reperfusion injury after ischemia (68), in pulmonary hypertension (111), and in hypercholesterolemia (31, 148). These data indicate that NOS activity is regulated by the abundance of its substrate and cofactor, although many more regulatory mechanisms exist in the cell, all of which contribute to the enzyme activity of eNOS (Fig. 2).

TRANSCRIPTIONAL REGULATION OF eNOS

Although the term "inducible" has been restricted to iNOS, eNOS expression is also regulated by a variety of stimuli. First, its basal expression is largely tissue dependent (151). eNOS is predominantly expressed in endothelial cells of large- and medium-sized blood vessels. In addition, there are numerous factors that affect the basal expression levels. For instance, fluid flow across the endothelium, also referred to as shear stress, upregulates eNOS expression (114, 115). Six shear stress-responsive elements have indeed been identified in the eNOS promotor sequence on cloning of the eNOS cDNA and identification of the promotor region (114, 141). Besides shear stress-responsive elements, the eNOS promotor also contains other putative *cis*-elements, including Sp1 and GATA motifs, a sterol regulatory element, estrogen-responsive elements, a nuclear factor-1 element, a cAMP-responsive element, and activator protein-1 (AP-1) and -2 (AP-2) binding sites (99, 134). Sp1 has been shown to be involved in lysophosphatidylcholine (lysoPC)-induced eNOS upregulation (25), whereas AP-1 activation mediates increases in eNOS expression in the presence of immunosuppresive drugs such as cyclosporin A (112). eNOS expression is also upregulated by cyclic strain (7),

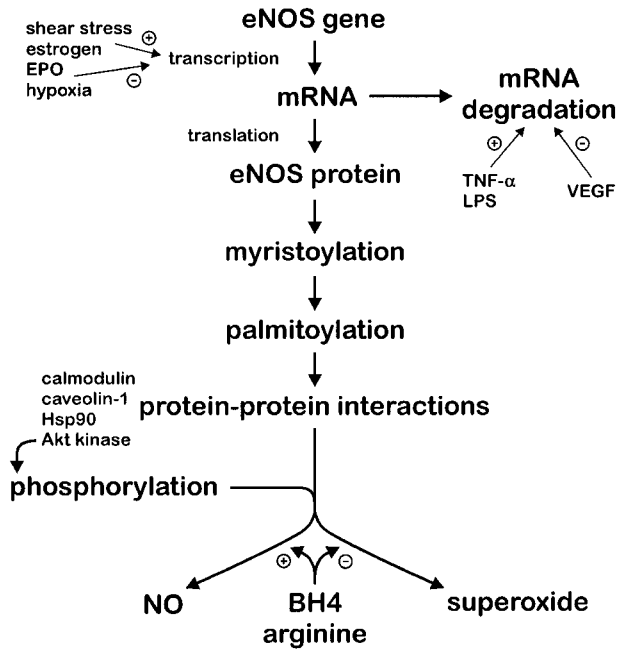

Fig. 2. Cellular events involved in the regulation of eNOS activity. The main pathway leading to a functional eNOS enzyme is depicted. For transcriptional regulation, (de)stabilization of eNOS mRNA, and protein-protein interactions, some examples of regulators of eNOS activity are shown. Once the enzyme is functional, the presence of substrate arginine and cofactor BH4 determines whether eNOS is producing nitric oxide (NO) or superoxide. EPO, erythropoietin; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; VEGF, vascular endothelial growth factor; Hsp90, 90-kDa heat shock protein.

agents that inhibit protein kinase C (PKC) (116), enhanced proliferative state (6), hydrogen peroxide (39), estrogen (150), vascular endothelial growth factor (VEGF) (13, 83), insulin (84), basic fibroblast growth factor (175), epidermal growth factor (175), transforming growth factor-β (TGF-β) (72), and low concentrations of oxidized LDL or its major atherogenic phospholipid lysoPC (67, 172). An increased eNOS expression by lysoPC and moderate amounts of oxidized LDL may implicate an endothelial antiatherosclerotic defense mechanism at the early stages of lesion formation.

Several factors that are known to lower eNOS expression include tumor necrosis factor-α (TNF-α) (114), erythropoietin (163), hypoxia (105), and high concentrations of oxidized LDL (88). In line with these regulatory mechanisms, renal eNOS expression is increased along with increases in cyclic strain as can be found in, e.g., hypertension secondary to lead or angiotensin II infusion (24, 156), whereas such an increase could not be found in erythropoietin-induced hypertension (113). Another determinant of eNOS expression is NO itself. NO has been shown to be involved in a negative-feedback regulatory mechanism and decreases eNOS expression via a cGMP-mediated process (157). In agreement, the decrease in glomerular filtration rate after administration of lipopolysaccharides could be attributable to inhibition of eNOS function, most likely by NO autoinhibition via activation of iNOS (139).

There is a marked discrepancy among amounts of eNOS mRNA, eNOS protein, and eNOS activity, demonstrating complex regulatory mechanisms at the posttranscriptional and posttranslational level. An important feature here that concerns posttranscriptional/posttranslational regulation is the stability (i.e., halflife) of the eNOS mRNA (12). mRNA levels represent the balance between gene transcription and mRNA degradation. The kinetics of mRNA degradation is dependent in part on nucleotide sequence motifs, which are usually located in the 3′ untranslated region of the gene. Possible interactions of specific proteins to these sequences may render the mRNA more or less susceptible to endonucleolytic cleavage. Two motifs often implicated in mRNA destabilization are present at the 3′ end of the eNOS mRNA (99). In accordance, some stimuli affect eNOS mRNA stability. TNF-α destabilizes eNOS mRNA, which is suggested to be mediated by the increased binding of regulatory cytosolic proteins to the 3′ untranslated region of the eNOS mRNA (3). Other stimuli that have been reported to decrease eNOS mRNA stability include lipopolysaccharides (endotoxins) (97), hypoxia (105), and oxidized LDL (91). Such mechanisms could also be involved in decreased eNOS expression in inflammatory models of renal injury such as necrotizing crescentic glomerulonephritis (65). On the other hand, certain conditions of shear stress upregulate eNOS mRNA levels through posttranscriptional events (176). VEGF- as well as hydrogen peroxide-induced eNOS upregulation are also dependent on an enhanced stability of eNOS mRNA (13, 39). In conclusion, eNOS expression is affected by various stimuli, which modify eNOS regulation at the mRNA level by inducing changes in transcription kinetics and stability of the eNOS mRNA.

COTRANSLATIONAL MODIFICATION OF eNOS: MYRISTOYLATION

In contrast to the other NOS isoforms, eNOS contains a myristoyl group that is covalently attached to the glycine residue at its NH_2 terminus. The turnover of the myristoyl group is as slow as that of eNOS itself, demonstrating the irreversibility of myristoylation (92). Myristoylation renders eNOS membrane bound, whereas iNOS and nNOS are predominantly, if not exclusively, cytoplasmic. The presence of eNOS at the membrane (especially at the plasma membrane) may serve an important purpose. It may bring eNOS in close proximity to factors that are required for its proper function, such as arginine, calcium, and cofactor BH4. It is of particular interest that arginine and calcium channels have indeed been identified in caveolae at the plasma membrane (51, 104). Studies in which the myristoylation site of eNOS was mutated have demonstrated that the myristoyl moiety is an absolute requirement for the membrane localization and activity of eNOS (136). Without this modification, eNOS is almost completely cytosolic and lacks palmi-

toyl moieties (94, 133). Most likely, myristoylation targets eNOS to the Golgi complex, where it is palmitoylated (140). Nevertheless, the NO-generating activity of the myristoyl-deficient enzyme in vitro is not impaired (44).

eNOS REGULATION AT THE POSTTRANSLATIONAL LEVEL

eNOS Palmitoylation

Degradation of eNOS is probably neither regulated nor modified by cellular responses, because eNOS has a rather long half-life of ~20 h (92). A posttranslational modification that does modify eNOS activity is palmitoylation. eNOS is palmitoylated on two cysteine residues near the NH_2 terminus (cysteine-15 and -26). This modification is reversible, requires eNOS myristoylation, stabilizes the association of eNOS with the membrane, and is required for a proper localization of eNOS (92). The steady-state turnover of the eNOS palmitoyl modification is 25 times faster than that of the myristoyl moiety or that of eNOS itself (92). The unique $(Gly\text{-}Leu)_5$ repeat between the two palmitoylation sites of eNOS is required for the palmitoylation (94). Agonists such as bradykinin have been suggested to promote eNOS palmitate turnover with a concomitant release of eNOS from the plasma membrane (133). Whether the transient depalmitoylation of eNOS is required for its agonist-induced activation is unclear. eNOS depalmitoylation is suggested to be mediated by the cytosolic enzyme acyl-protein thioesterase and to be potentiated by calcium-bound calmodulin (170). However, these bradykinin-induced phenomena were contradicted by others (92). The cellular enzyme activity of eNOS in which the palmitoylation sites were mutated was markedly decreased, but to a lesser extent than for the myristoyl-deficient enzyme (143). A mutated palmitoyl-deficient enzyme displays an altered cellular distribution compared with wild-type eNOS and is hardly detectable at the plasma membrane (57, 133). The reduced amount of NO generated by the palmitoylation-deficient enzyme within the endothelial cell was not caused by alterations in its catalytic properties, because purified wild-type and palmitoyl-deficient eNOS were kinetically identical (93). Again, these findings strongly indicate that the enzymatic activity of eNOS within the endothelial cell is dependent on its intracellular distribution.

eNOS Activation: Calcium vs. Tyrosine Phosphorylation

NOS differs from iNOS in that its activity is dependent on the presence of calcium and calmodulin. iNOS binds calmodulin very tightly so that calmodulin forms a constitutive subunit. eNOS activity is regulated by changes in the cytosolic calcium concentration and is therefore activated by hormones that induce a rise in intracellular calcium levels, such as bradykinin (62), estradiol (59), serotonin (17), VEGF (120), and histamine (79). Calmodulin binds the calcium, and the calcium-calmodulin complex interacts with eNOS, resulting in increased enzyme activity (see *eNOS Localization in Caveolae*).

On the other hand, eNOS activation by hemodynamic shear stress as well as isometric vessel contraction is independent of calcium (8, 49). The exact mechanism behind this mechanochemical transduction process is not completely understood. Shear stress-induced eNOS activation is regulated by a potassium channel, which might act as a mechanochemical transducer within the plasma membrane of the endothelial cell (27, 118), whereas it is not involved in eNOS activation by calcium-mobilizing agents (69). Other cell components that play a role in shear stress-induced eNOS activation include caveolae and the cytoskeleton (70, 132). The endothelial cytoskeletal network usually maintains the shape of the endothelial cell, which is essential for the relative impermeability of the endothelial lining of the vessel wall. An alteration in fluid flow across the endothelium results in a change in the tension of the endothelial cytoskeleton and in the transmission of this signal throughout the cell, which immediately modulates eNOS activity. Accordingly, disruption of the cytoskeleton attenuates flow-induced eNOS activity, although it does not affect agonist-induced calcium-dependent NO generation (70). Interestingly, NO is important for stability of the endothelial cytoskeleton and therefore has a role in the relative impermeability of the endothelium (85, 96). In contrast, under certain conditions (e.g., hypoxia) and dependent on the vascular bed, endothelial permeability is enhanced due to VEGF-induced increases in NO levels (47). Furthermore, shear stress-induced eNOS activation is abrogated in the presence of tyrosine kinase inhibitors, suggesting that tyrosine phosphorylation is involved (8, 28). The tyrosine kinase involved in this process might be the VEGF receptor Flk-1, because this receptor is phosphorylated and associated with the adaptor protein Shc on application of shear stress (21). Shear stress, but also calcium-mobilizing agents, activate protein tyrosine kinases and result in enhanced tyrosine phosphorylation of specific proteins of 42 and 44 [mitogen-activated protein (MAP) kinases] and proteins of 88, 90, 103, and 114 kDa (8, 22). Moreover, estradiol- and VEGF-induced increases in eNOS activity are attenuated by tyrosine kinase inhibitors (22, 120), suggesting that tyrosine phosphorylation may be required for eNOS activation by both shear stress and calcium-mobilizing agents.

The protein tyrosine phosphatase inhibitor phenylarsine oxide has been shown to activate eNOS in the presence and absence of extracellular calcium and the calmodulin inhibitor calmidazolium, which is in accordance with the other indications that suggest that tyrosine phosphorylation is essential for calcium-independent eNOS activation (48). The phenylarsine oxide-induced effect is inhibited by the protein tyrosine kinase inhibitor erbstatin. Also, ceramide enhances eNOS activity in a calcium-independent manner (71). Whether this is accompanied by an increase in eNOS phosphorylation or is dependent on tyrosine phosphor-

ylation of other proteins is not known. Recently, there have been some indications that eNOS might be phosphorylated on tyrosine residues, although this is suggested to be dependent on the amount of cell passages, indicating that the intracellular mechanisms that regulate tyrosine phosphorylation of eNOS are rapidly lost when cells are cultured (48, 55). This may have been the reason why many others have failed to detect tyrosine phosphorylation of eNOS (28, 38, 159, 160).

eNOS Serine Phosphorylation

eNOS is phosphorylated on serine residues on exposure of endothelial cells to shear stress as well as to calcium-mobilizing agents (28, 108). In vitro, eNOS can be phosphorylated by PKC and cAMP-dependent kinase [protein kinase A (PKA)] (23, 66). In addition, PKC-activating phorbol esters induce eNOS phosphorylation in intact cells, which is accompanied by a decrease in eNOS activity. This decrease is prevented by PKC inhibitors, suggesting that eNOS activity may be regulated by PKC-mediated eNOS phosphorylation in vivo. PKA mediates phosphorylation at threonine-495 and serine-1177 of human eNOS in vitro in a calcium- and calmodulin-independent manner, resulting in eNOS activation (109). Furthermore, there have been some indications suggesting that PKA might be involved in eNOS activation in vivo (152).

Recently, it was shown that the serine/threonine kinase Akt (also known as protein kinase B) phosphorylates eNOS in vitro as well as in vivo, thereby activating the enzyme (52, 53). Akt-mediated eNOS phosphorylation was suggested to play a role in both calcium-dependent and -independent eNOS activation pathways. Both shear stress and VEGF induce phosphorylation and activation of Akt (38). LY-294002 and wortmannin, inhibitors of phosphatidylinositol 3-kinase, the upstream activator of Akt, reduce shear stress-, VEGF-, and insulin-induced phosphorylation and activation of eNOS (38, 53, 174). In accordance, the use of activation-deficient Akt has demonstrated that Akt is required for VEGF- and insulin-mediated eNOS activation (52, 173). The site of phosphorylation by Akt is serine-1177 in human eNOS (52). Shear stress-induced Akt activation is unaffected by the removal of calcium or by the calmodulin antagonist calmidazolium, implying that Akt-mediated eNOS activation in response to shear stress requires only trace amounts of calcium (in a similar way to iNOS) (38). This has been confirmed by Sessa and co-workers (103), whose studies implicated that Akt-mediated eNOS phosphorylation reduces the dissociation of calmodulin from activated eNOS. Shear stress-induced serine phosphorylation of eNOS was shown to be inhibited by tyrosine kinase inhibitors, indicating that tyrosine phosphorylation of a regulatory protein, which is activated upstream of Akt, precedes Akt-mediated eNOS phosphorylation (28).

Bradykinin also induces serine phosphorylation of eNOS, which is maximal after 5 min and is prolonged for 20 min. The serine-phosphorylated enzyme is primarily cytosolic. This phosphorylation event appears to be calcium dependent, because it is inhibited by calmodulin antagonists and by removal of extracellular calcium (108, 109). Whether bradykinin-induced serine phosphorylation of eNOS is mediated by Akt and whether this is required for eNOS activation remain to be determined. Given the fact that eNOS-mediated NO generation occurs faster than bradykinin-induced serine phosphorylation of eNOS, it is likely that this phosphorylation event is involved in the downregulation of eNOS activity. Accordingly, incubation of endothelial cells with NO donors induces serine phosphorylation of eNOS, while inhibiting its activity (19, 108). This suggests that eNOS-generated NO may induce phosphorylation of eNOS in vivo and therefore may act as a negative feedback mechanism for eNOS activity. If this is the case, PKC might be the kinase involved in this inhibitory phosphorylation event, because PKC-mediated eNOS phosphorylation is known to inhibit eNOS activity (66). Furthermore, the involvement of PKC in eNOS inactivation is suggested by the fact that G protein-coupled receptors, including the receptors for bradykinin, acetylcholine, serotonin, and histamine activate phospholipase C, an upstream activator of PKC (16, 41).

Finally, other kinases could be involved as well in the regulation of eNOS activity, perhaps dependent on the agonist. For instance, studies in which the MAP kinase kinase inhibitor PD-98059 was used have suggested that members of the MAP kinase family play a role in estradiol-induced eNOS activation (22). Whether these kinases are actually able to phosphorylate eNOS itself has not been examined.

In summary, serine phosphorylation of eNOS is essential for the cellular regulation of eNOS function. It might either increase or decrease its activity, depending on the kinase and the serine residue involved. In particular, the serine/threonine kinase Akt has been shown to regulate eNOS activity in vitro as well as in vivo.

eNOS Localization in Caveolae

Studies from many research groups have indicated that the localization of eNOS within the cell determines its activity. One particular site in the cell that apparently is of importance to eNOS function is the caveolus. Caveolae are specialized invaginations of the plasma membrane and are present in most cell types, with the highest number being present in endothelial cells, adipocytes, fibroblasts, and smooth muscle cells. The main components of caveolae are cholesterol, glycosphingolipids, and some structural proteins, such as caveolin, whereas phospholipids are practically absent (121, 144). This membrane domain harbors many signal transduction pathways, and evidence is accumulating that signal transduction pathways ascending from various stimuli from outside of the cell converge at this specific spot (4, 95). eNOS was reported to be present in caveolae but not in other parts of the plasma membrane (143). However, it must be taken into account

that the experimental procedures used for the isolation of caveolae might influence the experimental findings. For instance, detergent-based caveolae isolation procedures have not always allowed eNOS coisolation, although other caveolae isolation methods by the same researchers did show that eNOS was localized within caveolae (143). Similar findings have been described for the epidermal growth factor receptor (110, 164) and for the insulin receptor (64). Unfortunately, most of these studies fail to show by immunoelectron microscopic analysis of intact cells or of isolated caveolae that these particular proteins do reside in caveolae. In addition, the results of the biochemical experiments are difficult to interpret, because the presence of alternative detergent-insoluble membrane domains that are not homologous to caveolae has been demonstrated (50, 61). Thus, although present in caveolae, the presence of eNOS in other plasma membrane domains cannot be excluded.

The localization of eNOS within caveolae renders the enzyme inactive. In caveolae, eNOS activity is inhibited by caveolin-1 (77). Caveolin-1 is not exclusively located in caveolae but is also resident in the Golgi complex and is known to bind to the caveolae-enriched lipids cholesterol and glycosphingolipids (26). Caveolin-1 is an intrinsic membrane protein (although not membrane spanning) that is irreversibly palmitoylated and not only binds to itself in an oligomeric complex but also interacts with eNOS via its so-called scaffolding domain (amino acid residues 82–101) (77, 107). In addition, caveolin-1 may contain a second eNOS-binding motif (56, 77). Interestingly, within the endothelial cell the binding of eNOS to caveolin-1 requires both myristoylation and palmitoylation of eNOS, whereas in vitro the eNOS-caveolin-1 interaction is independent of both acyl groups (56). This indicates that the acyl modifications mediate caveolar localization rather than caveolin-1. The interaction between eNOS and the caveolin-1 scaffolding domain strongly reduces eNOS activity because caveolin-1 interferes with the binding of calmodulin to eNOS when cytosolic calcium levels are low (77, 106). However, the interaction with the scaffolding domain is not restricted to eNOS. Other signaling proteins that interact with the caveolin-1 scaffolding domain include phosphatidylinositol 3-kinase, c-Src, and Ha-Ras (29, 89). Inhibitory interactions have been reported for the PDGF receptor (168), epidermal growth factor receptor (30), nerve growth factor receptor Trk A (10), MAP kinases (40), and G protein α-subunits (90). The sequence in human eNOS that mediates the interaction with caveolin-1 is ^{350}**F**SAAPFSG**W**^{358}, which corresponds to the scaffolding domain-binding consensus sequences φ*X*φ*XXXX*φ and φ*XXXX*φ*XX*φ (φ represents Trp, Phe, or Tyr; *X* represents any amino acid) (29, 56). The activity of eNOS in which this domain was mutated was not affected by caveolin-1, in contrast to wild-type eNOS (56). In accordance, an increased caveolin-1 expression results in an increased eNOS-caveolin interaction and in reduced eNOS activity (43, 44). The scaffolding domain-binding motif is situated in the eNOS oxygenase domain, although an interaction between caveolin-1 and the reductase domain of eNOS may also exist (58). To which residues of the reductase domain caveolin-1 might bind is presently unknown.

Caveolae-localized eNOS also interacts with the bradykinin receptor and with the cationic amino acid transporter CAT-1 (76, 104). CAT-1 is involved in the transfer of the NOS substrate arginine across the membrane. Receptors for estrogen (80) and VEGF (42) as well as a calcium pump (51) are also present in caveolae. The receptors for bradykinin and acetylcholine are not constitutively present in caveolae but may translocate to caveolar membranes on agonist stimulation (36, 46). This indicates that most of the components required for a proper eNOS function are concentrated within the caveolae, which may facilitate eNOS function. However, the molecular interactions among eNOS, CAT-1, and G protein-coupled receptors may need further investigation, because the techniques used in these studies imply the presence of these proteins in caveolae rather than a direct interaction among these molecules due to the detergent insolubility of caveolae. Some of these molecular interactions have now been confirmed in in vitro assays (98). Moreover, the interaction of eNOS with the bradykinin receptor blocks the electron transfer within the eNOS molecule without affecting the binding of its substrate arginine and cofactor BH4 (60).

In the presence of shear stress (131) or calcium-mobilizing agents such as bradykinin (127), acetylcholine (45), estradiol (80), and calcium ionophore (45), calcium-bound calmodulin associates with eNOS, whereas caveolin-1 is displaced. The calcium ions that are mobilized into the cytosol are reported to originate from the extracellular environment and from intracellular calcium stores (i.e., endoplasmic reticulum) (32, 62). Caveolin-1 displacement may coincide with eNOS depalmitoylation (133). As a consequence of both of these processes, eNOS is released from the plasma membrane into the cytosol (108). However, this model could not be confirmed by all research groups (92). Interestingly, Venema et al. (160) reported an increased interaction of eNOS and caveolin-1 on bradykinin incubation of endothelial cells. How eNOS is inactivated is unclear. An increase in Golgi-localized eNOS after its activation has been demonstrated (127). Again, this phenomenon could not be confirmed by others (48). Probably, the activated eNOS-calmodulin complex generates NO until intracellular calcium levels drop to the point that calmodulin dissociates and the inhibitory eNOS-caveolin complex reforms (45). This is not unlikely because the agonist-induced increases and subsequent decreases in intracellular calcium and NO show similar kinetics (18).

Because cholesterol is one of the main constituents of caveolae and since hypercholesterolemia has been associated with a change in NO production (20), several laboratories have studied the effect of endothelial cholesterol loading on eNOS activity. Endothelial cells incubated in the presence of cholesterol displayed a 50% increase in NO release in response to calcium

ionophore, whereas eNOS expression was increased to the same extent (123). Caveolin expression was not significantly increased in these experiments, whereas the number of caveolae was also increased by 50%. In contrast, incubations with higher amounts of cholesterol inhibited eNOS activity (34). Incubation of endothelial cells with agents that lower the cholesterol content of caveolae such as oxidized LDL and cyclodextrin resulted in translocation of both caveolin-1 and eNOS from the caveolae and in inhibition of acetylcholine-induced eNOS activation (11). Myristoylation, palmitoylation, and phosphorylation of eNOS were not affected by cholesterol depletion, implying that these molecular modifications were not involved in the eNOS inactivation. Thus, although caveolar eNOS itself is inactive, caveolar localization of eNOS is required for its activity because conditions that inhibit the localization of eNOS in caveolae (e.g., inhibition of eNOS myristoylation and cholesterol depletion) markedly decrease eNOS activity. This implies that eNOS needs to be localized in caveolae to be able to become activated.

eNOS in the Golgi Complex

Although most studies apparently agree in that NO generation requires a functional eNOS enzyme at the plasma membrane, and in particular at the caveolae, most of the cellular eNOS is contained within the Golgi apparatus in cultured endothelial cells as well as in intact blood vessels (5, 140). The first 35-amino acid residues of eNOS, containing all three acylation sites, are required and sufficient for the targeting of eNOS to the Golgi complex (94). Whether eNOS at the Golgi is bound to caveolin-1 in a similar complex as has been described for caveolae is unknown. At present, no clear suggestion has been presented on the function of the Golgi localization of eNOS. Golgi localization might be required for eNOS repalmitoylation after activation and depalmitoylation at the plasma membrane. Palmitoyl transferases may be present in the Golgi complex. Accordingly, eNOS would recycle between the plasma membrane and the Golgi complex. In that case, eNOS probably moves from the Golgi to caveolae via vesicular transport, as has been described for caveolin-1 (86). However, data supporting this hypothesis are lacking due to the few immunoelectron microscopic studies that have been presented so far.

Incubation of cerebrovascular endothelial cells with the Golgi complex-disrupting agent brefeldin A inhibited NOS activity, implying that the Golgi localization of eNOS is essential for eNOS activity, although it does not provide any evidence that Golgi-localized eNOS may be directly activated (146). Probably, Golgi disruption also indirectly affects eNOS localization at the plasma membrane, because eNOS is thought to recycle between the Golgi complex and the plasma membrane. However, it cannot be completely excluded that the Golgi might be a site of NO production. That Golgi-localized eNOS is not a "silent" pool of eNOS is evident from studies in which examination of recovery after photobleaching of fluorescent-labeled eNOS showed that the enzyme is translocating to and from the Golgi complex at high speed (145).

Interaction with the Cytoskeleton

Within the endothelial cell, eNOS has been reported to reside in a detergent Triton X-100-insoluble cell fraction under resting conditions (57). However, others have reported that eNOS translocates to a Triton X-100-insoluble cell fraction on stimulation with the calcium-mobilizing agent bradykinin (159). At the same time, bradykinin induces tyrosine phosphorylation of cellular proteins, which apparently is required for translocation of eNOS because tyrosine kinase inhibitors block the translocation. Treatment of endothelial cells with bradykinin also induces a transient increase in the amount of detergent-insoluble caveolin-1 (160). Again, the translocation could be inhibited by tyrosine kinase inhibitors.

Translocation of eNOS into a detergent-insoluble cell fraction is also induced by shear stress (48). Shear stress-induced eNOS activation as well as the concomitant translocation of eNOS into detergent-insoluble membranes were dependent on the presence of tyrosine kinase inhibitors. Whether caveolin-1 also translocates to a detergent-insoluble membrane fraction after shear stress has not been determined. In addition, eNOS activation by the tyrosine phosphatase inhibitor phenylarsine oxide also coincided with a translocation of both eNOS and caveolin-1 from a detergent-soluble to a detergent-insoluble cell fraction (48, 160). This translocation was independent of calcium.

Although this detergent-insoluble cell fraction has been referred to as cytoskeleton associated (87, 166), one must not forget that caveolae are also detergent insoluble (169). In addition, other distinct microdomains have been reported that are Triton X-100 insoluble and enriched in glycosyl phosphatidylinositol-anchored proteins (138). Furthermore, depending on the experimental procedures, this translocation may also account for an interaction between caveolae and the endothelial cytoskeleton, providing a possible explanation as to why both eNOS and caveolin-1 translocate to detergent-insoluble membrane domains.

Additional Interactions

A tyrosine-phosphorylated protein of 90 kDa interacts with eNOS in cultured endothelial cells (159). The interaction is markedly enhanced within 1 min of bradykinin stimulation. The associated protein was denominated eNOS-associated protein-1 (ENAP-1). At present, it is unknown whether this protein interacts directly or indirectly with eNOS or whether ENAP-1 associates with the detergent-insoluble cell fraction to which eNOS translocates on bradykinin stimulation, as was reported by the same research group (159). Although bradykinin did induce a translocation of a substantial amount of eNOS into the detergent-insoluble cell fraction, the eNOS-ENAP-1 interaction was detected in the Triton X-100-soluble cell fraction.

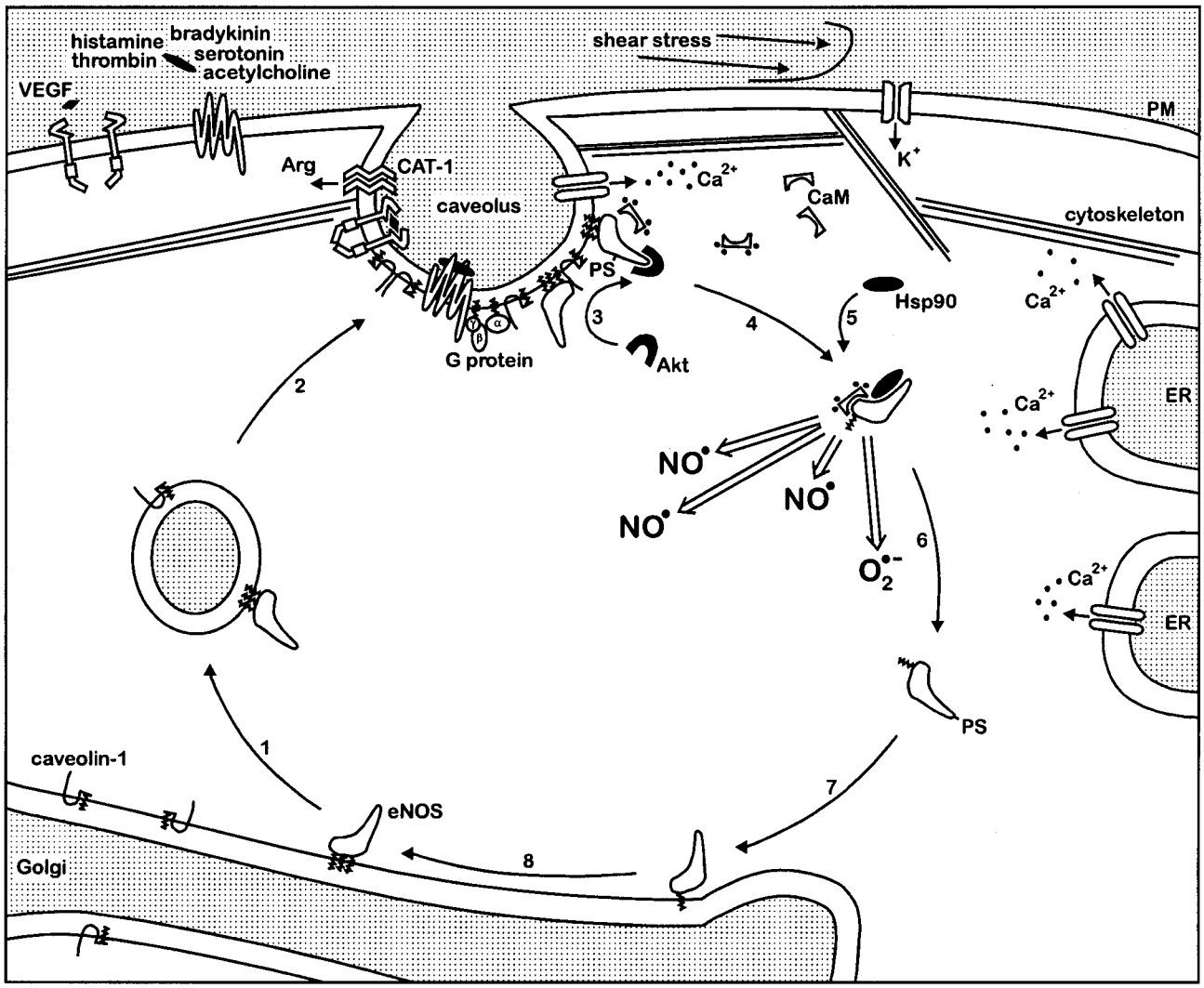

Fig. 3. The eNOS activation-deactivation cycle (*1–8*). eNOS, which is resident in the Golgi complex (anchored in the membrane by 1 myristoyl and 2 palmitoyl groups), is transported together with caveolin-1 to the caveolae at the plasma membrane (PM) via vesicles (*1* and *2*). Within the caveolae, eNOS is bound to caveolin-1, which inhibits eNOS activity. Shear stress signals via a potassium channel and the cytoskeleton, which results in tyrosine phosphorylation of specific proteins, activation of phosphatidylinositol 3-kinase, and subsequently in activation of Akt kinase. Akt activation by shear stress but also by VEGF activates eNOS by serine phosphorylation (PS; *3*), which increases the affinity of eNOS for calmodulin. After agonist binding at the plasma membrane, eNOS-activating receptors translocate to caveolae. The 7-membrane-spanning domain-containing receptors activate G proteins, whereas the VEGF receptor signals via its tyrosine kinase domain. These receptors activate calcium channels at the caveolae. Furthermore, they activate calcium channels of the endoplasmic reticulum (ER) via phospholipase C and inositol 1,4,5-trisphosphate. This calcium flux induces binding of calmodulin to eNOS, whereas the eNOS-caveolin-1 interaction is disrupted. At the same time, eNOS is depalmitoylated (only the myristoyl moiety remains) and therefore translocates into the cytosol (*4*). On binding of calmodulin, eNOS generates NO (and under certain conditions superoxide, $O_2^{-}\cdot$), which may be enhanced by the interaction with Hsp90 (*5*). eNOS is inactivated by PS and by the dissociation of calmodulin (*6*). Perhaps phosphorylation induces the eNOS-calmodulin dissociation. After translocation to the Golgi complex (*7*), eNOS is repalmitoylated (*8*), which enables eNOS to be transported to the caveolae again. CAT-1, cationic amino acid transporter.

Another protein that has been demonstrated to interact with eNOS is the 90-kDa heat shock protein (Hsp90) (54, 142). This protein belongs to the family of heat shock proteins, which affect activity and function of other proteins by acting as molecular chaperones, thereby modulating their structure. These heat shock proteins were first identified for their role in physiological stress situations (e.g., heat), but their role in common molecular regulatory mechanisms has nowadays been beyond debate. Other signaling proteins to which Hsp90 binds include G protein subunits, MAP kinase kinase, Src, and Raf (128). Binding of Hsp90 to eNOS in response to histamine, VEGF, or shear stress increases eNOS activity by facilitating the calmodulin-induced displacement of caveolin from eNOS (63). This increase in eNOS activity is inhibited by the Hsp90

inhibitor geldanamycin (54). Given the fact that Hsp90 and ENAP-1 are similar in their molecular weight, it could very well be possible that ENAP-1 is Hsp90. Whether eNOS-associated Hsp90 is indeed tyrosine phosphorylated remains to be shown.

Studies using purified eNOS and phospholipid vesicles have shown that eNOS is also able to bind phospholipids. This association is restricted to anionic species such as phosphatidylcholine. Ohashi et al. (117) showed that these phospholipids enhanced eNOS activity. In contrast, Venema and co-workers (158) showed a decrease in its activity. They demonstrated that the binding of eNOS to phosphatidylcholine vesicles prevented the interaction of the enzyme with calmodulin. Calmodulin and the phospholipids bound eNOS at the same site. Whether the association of eNOS with phospholipids has some physiological relevance remains to be shown. It is difficult to envision a role for phosphatidylcholine in the regulation of eNOS, because caveolar membranes are virtually devoid of phospholipids (122). Perhaps phospholipids prevent eNOS activation in the Golgi complex. Another lipid molecule that binds eNOS and modifies its activity in vitro is oleic acid (33). Whether this fatty acid has some significant role in eNOS regulation in vivo also remains to be determined.

CONCLUDING REMARKS

There is a large and rapidly expanding amount of data on eNOS cell biology. A model based on the published eNOS data is depicted in Fig. 3. However, still very little is known about the exact eNOS regulation within the cell. The most important reason for this is that a considerable amount of data generated by different groups is contradictory. Many conclusions that are drawn from cell-free in vitro systems may not turn out to be true for the in vivo situation. Similarly, certain interactions may not exist in vivo but might be introduced during the experimental procedures, which are used for studying the in vivo material. Cell culture techniques will also definitely have their impact on eNOS function and regulation (48, 149). Furthermore, insight into the mechanisms of regulation are impeded by the fact that eNOS expression and regulation might be dependent on the vascular bed, from which the endothelial cells are derived. This is reflected by the different localization of eNOS within various vessel types (5). In addition, different vessel types also display a different number of caveolae, suggesting that the kinetics of eNOS activation is dependent on the origin of the endothelium. With regard to the kidney, regulatory information is almost absent despite the crucial role of eNOS in normal renal physiology.

In general, eNOS activity is first of all modulated by the presence of its substrates and cofactors within the cell. These factors determine whether eNOS is a NO- or superoxide-producing enzyme. In addition, eNOS activity is affected by agents and conditions that affect its expression and by its attachment to the membrane, its cellular localization, phosphorylation events, and multiple protein-protein interactions. Of course, these determinants are highly interlinked within the cell and provide a complex regulatory network. The main challenge now will be to unravel this network.

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