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Review

Signal transduction of eNOS activation

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Abstract

Consistent with its classification as a Ca^{2+}/calmodulin-dependent enzyme the constitutive endothelial nitric oxide (NO) synthase (eNOS) can be activated by receptor-dependent and -independent agonists as a consequence of an increase in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) and the association of the Ca^{2+}/calmodulin complex with eNOS. Additional post-translational mechanisms regulate the activity of eNOS, including the interaction of eNOS with caveolin-1, heat shock protein 90 (Hsp90), or membrane phospholipids, as well as enzyme translocation and phosphorylation. In response to fluid shear stress the maintained production of NO by native and cultured endothelial cells is associated with only a transient increase in $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+} and in the presence of calmodulin antagonists, shear stress stimulates a maintained production of NO which is insensitive to the removal of extracellular Ca^{2+}, but sensitive to tyrosine kinase inhibitors, Hsp90-binding proteins and phosphatidylinositol 3-kinase inhibitors. A pharmacologically identical activation of eNOS can be induced by protein tyrosine phosphatase inhibitors suggesting that the phosphorylation of eNOS, and possibly that of an associated regulatory protein(s), is crucial for its Ca^{2+}-independent activation.

Keywords: Endothelial function; Nitric oxide; Signal transduction

1. eNOS

Nitric oxide: Since the discovery of an endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki [1], which was later identified as nitric oxide (NO) [2–4], it has become clear that there are a number of additional endothelium-derived vasodilator and vasoconstrictor autacoids (endothelin-1, prostacyclin, prostaglandin H_2 and the endothelium-derived hyperpolarizing factor: EDHF). None of these autacoids play such a central role in the regulation of vascular tone and homeostasis as the principle EDRF, the free radical NO, which is generated via a five-electron oxidation of a guanidino nitrogen from L-arginine.

The nitric oxide synthase (NOS) present in vascular endothelial cells is a multi-domain enzyme consisting of an N-terminal oxygenase domain (amino acids 1–491) that contains binding sites for heme, L-arginine (Glu361) [5] and tetrahydrobiopterin (H_4B), and a reductase domain (amino acids 492–1205) containing binding sites for FMN, FAD, NADPH and calmodulin (CaM) [6]. The functional endothelial NO synthase (eNOS), like the other isoforms, is a dimer comprised of two identical subunits, both of which are myristoylated and palmitoylated. H_4B may well prove to be essential for the dimerisation of eNOS as NOS monomers are both catalytically inactive and unable to bind either L-arginine or H_4B [7]. Only the dimer retains the ability to bind substrate and cofactor and the presence of H_4B is critical for dimer formation [8]. Maintenance of the integrity of the H_4B-binding site on the eNOS oxygenase domain appears to involve a zinc tetrathiolate or Zn[*S*-cysteine]$_4$ positioned equidistant from each heme and H_4B [9]. The identification of ZnS_4 in eNOS may well be of physiological relevance and it is feasible that increasing nitrosative stress in vivo may result in the release of the zinc from the oxygenase domain, destabilise the H_4B and/or deplete eNOS of this essential cofactor and favour the generation of superoxide anions (O_2^-).

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During the synthesis of NO, NADPH-derived electrons pass into the reductase domain flavins and then must be transferred to the heme located in the oxygenase domain so that the heme iron can bind O_2 and catalyse stepwise NO synthesis from L-arginine [10–14]. The binding of CaM to its binding site (amino acids 493–512) is generally accepted to activate NO synthesis by enabling the reductase domain to transfer electrons to the oxygenase domain [10,15].

Comparative analysis of NOS domain interactions shows that subunit association of eNOS involves not only head to head interactions of oxygenase domains but also tail to tail interactions of reductase domains and head to tail interactions between oxygenase and reductase domains [16]. While, detailed experiments have not been performed on eNOS, domain swapping occurs in iNOS dimers, suggesting that in order to properly align reductase and oxygenase domains for NO synthesis electrons are transferred from the flavin to heme of adjacent subunits [17] (see Fig. 1).

Superoxide anion (O_2^-): In addition to NO, all of the NOS isoforms can generate O_2^- in a Ca^{2+}/calmodulin-dependent manner, especially in the absence of L-arginine and H_4B [18–22]. While some investigators initially attributed the ability of eNOS to generate O_2^- to an artefact associated with endothelial cell culture, i.e. H_4B depletion in passaged cells, evidence is continuously accumulating to suggest that H_4B depletion, and thus an eNOS-dependent generation of O_2^-, could be implicated in hypertension and atherosclerosis. Indeed, H_4B can be oxidised by peroxynitrite ($ONOO^-$), to dihydrobiopterin such that $ONOO^-$ may attenuate eNOS activity by essentially depleting endothelial cells of H_4B. Under these conditions, eNOS Ca^{2+}-dependently produces O_2^- rather than NO [22] and amplifies intracellular oxidative stress. While the possibility that eNOS-derived O_2^- contributes to endothelial dysfunction has been much debated over the last few years [23,24], such an effect could account for the observation that H_4B restores endothelial function in hypercholesterolemic patients [25]. Given that the redox state of endothelial cells and for example the activation of redox-sensitive transcription factors is regulated by the balance between NO and O_2^- production, eNOS may well be the most crucial enzyme determining the anti- or pro-hypertensive and eventually pro-atherogenic state of the vasculature.

2. Ca^{2+}-dependent eNOS activation

2.1. The interaction of eNOS with calmodulin

The eNOS is classified as a constitutive and strictly Ca^{2+}/CaM-dependent enzyme [26]. Indeed, an increase in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$), as observed following receptor-dependent and -independent agonist stimulation, enhances endothelial NO production and elicits vasodilatation (for review, see Ref. [27]). Both the agonist-induced NO formation and subsequent vasodilatation are abolished by the removal of Ca^{2+} from the extracellular fluid [28,29]. The identification of a CaM-binding domain in the primary structure of eNOS [30–32] together with the finding that CaM binding proteins inhibited enzyme activity [26] suggested that the binding of a Ca^{2+}/CaM complex is essential to activate the constitutive enzyme.

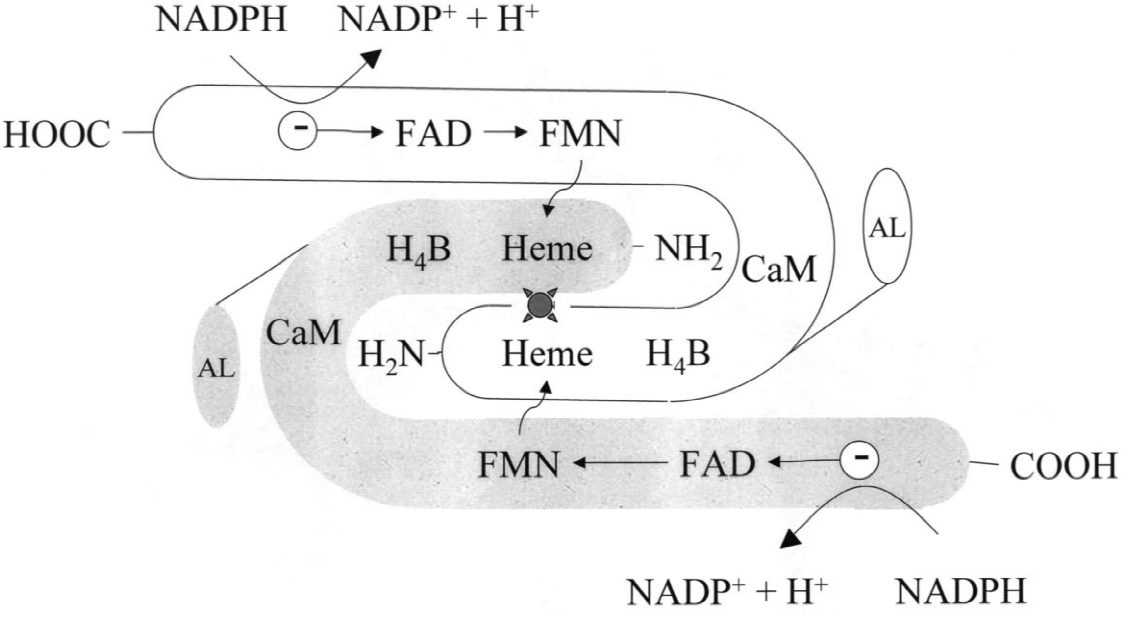

Fig. 1. Proposed model for an eNOS dimer indicating domain swapping and electron transfer pathway. CaM, calmodulin; H_4B tetrahydrobiopterin; AL, auto-inhibitory loop, ✱ represents the ZnS_4 recently described to be positioned equidistant from the two heme groups and H_4B.

Alterations in the CaM-binding domain determine the Ca^{2+} sensitivity of the various NOS isoforms and substitution of eNOS and iNOS CaM-binding domains in eNOS/iNOS chimeric proteins produces major alterations in the Ca^{2+}/CaM dependence of the intact enzymes. Replacement of the iNOS sequence with that of eNOS creates a functional enzyme which is both Ca^{2+}- and CaM-dependent, while replacement of the eNOS CaM-binding sequence with that of iNOS creates a functional enzyme which is CaM-independent but that remains Ca^{2+}-dependent [33]. The CaM-binding domain of eNOS has also been reported to directly bind phospholipids, and thus may determine the membrane association of the enzyme. Both a direct physical interaction and an association dependent on the phosphorylation of both the CaM-binding domain of eNOS and CaM have been proposed [34,35].

CaM may however bind to more than one site in a NOS subunit, a finding emphasised by more recent studies on the interaction between CaM and caveolin-1 [36,37]. In nNOS, and probably also in eNOS, CaM effects an electron transfer at two separate sites within one subunit. Not only does CaM control NO synthesis by governing heme iron reduction, it also enhances reductase activity by two mechanisms, only one of which is associated with an increased rate of flavin reduction [36].

Another distinctive difference between the Ca^{2+}-regulated and Ca^{2+}-independent NOS isoforms is the existence of a unique polypeptide (45 amino acid) insert in the FMN binding domains of the Ca^{2+}-dependent enzymes which is not shared by iNOS or other related flavoproteins [38].Three-dimensional molecular modelling suggested that the insert originates from a site immediately adjacent to the CaM-binding sequence and synthetic peptides derived from the 45 amino acid insert were found to potently inhibit the binding of CaM to eNOS as well as enzyme activity [38]. Based on these observations, it was suggested that the polypeptide insert is an auto-inhibitory control element, docking with a site on eNOS which physically impedes CaM binding and thus enzyme activation. Such a control mechanism would imply that CaM must displace the insert on binding to eNOS. The insert peptide is also a potential site for phosphorylation as 12 of the 45 amino acids are either serine or threonine [38], thus, phosphorylation and/or dephosphorylation may influence the affinity of insert peptides for binding and the sensitivity and/or affinity of eNOS/CaM binding.

2.2. The interaction of eNOS with caveolin-1

Caveolae, are invaginations of the plasma membrane which function to bind and organise a variety of signalling molecules [39]. The chief structural component of caveolae are cholesterol and structural proteins, such as the caveolins (caveolin-1, -2 and -3). Caveolin-1 is abundant in endothelial cells and its hairpin structure can be attributed to the inclusion of a 33-residue membrane spanning region between the N- and C-terminal cytosolic domains. A component of the C-terminal membrane proximal segment, termed the scaffolding domain (amino acids 82–101) is responsible for attachment of a number of signal proteins to the caveolae, including G protein subunits, phosphatidylinositol 3-kinase and the Src family protein tyrosine kinases [40,41].

The subcellular localisation of eNOS and alterations in its cellular compartmentalisation following cell stimulation are controversial. Previous reports have assigned eNOS to the Golgi apparatus [42–44], while others have localised eNOS in plasma membranes [45] or partially/exclusively in plasmalemmal caveolae [46–49]. The truth lies somewhere in between as immunostaining of porcine coronary arteries for eNOS reveals an association with the plasma membrane and with a perinuclear region, identified as the Golgi apparatus. Moreover, co-staining of endothelial cells with antibodies raised against eNOS and caveolin-1 clearly shows that not all eNOS is co-localised with caveolin-1 (Fig. 2). In which fraction eNOS is active in unstimulated cells, and can account for the basal production of NO is also controversial, since the eNOS in caveolae is thought to be mostly inactive (see below) and disruption of the Golgi apparatus in rabbit carotid arteries failed to affect NO-mediated relaxation [50].

The binding of caveolin-1 to a consensus site ($\mathbf{F}_{350}$XAAP**F**XXW) in the eNOS oxygenase domain is proposed to antagonise CaM binding and thereby inhibit enzyme activity [41,51–53]. This motif in NOS lies between the heme and the CaM-binding domain adjacent to a glutamate residue which is necessary for the binding of L-arginine, suggesting that caveolin-1 may interfere with heme iron reduction, similar to the L-arginine analogues [53]. Analysis of the crystal structure of the eNOS oxygenase domain revealed that the putative caveolin-1 recognition motif is primarily inaccessible to solvent. Therefore, it is relatively unlikely that caveolin-1 is able to interact with this sequence [9]. Other groups have however detected interaction between both the N- and C-terminal domains of caveolin-1 and the oxygenase domain of eNOS [53,54]. In experiments to examine how a caveolin-1 scaffolding domain peptide (amino acids 82–101) would affect NO synthesis, it was recently demonstrated that caveolin-1 must bind to the reductase domain of eNOS in order to inhibit enzyme activity [37]. Therefore, caveolin-1 binding to the reductase domain of eNOS compromises its ability to bind CaM and to donate electrons to the heme subunit, thereby inhibiting NO synthesis [37]. The interaction of caveolin-1 with the reductase domain was independent of the caveolin binding motif and reversed by CaM. Co-expression of eNOS and caveolin-1 in COS-7 cells leads to a marked inhibition of enzyme activity [55]. This inhibitory interaction is reported to be reversed by the addition of CaM, suggesting that eNOS activity may be determined by the relative proportion of eNOS–Ca^{2+}/CaM to eNOS–caveolin-1-binding [55].

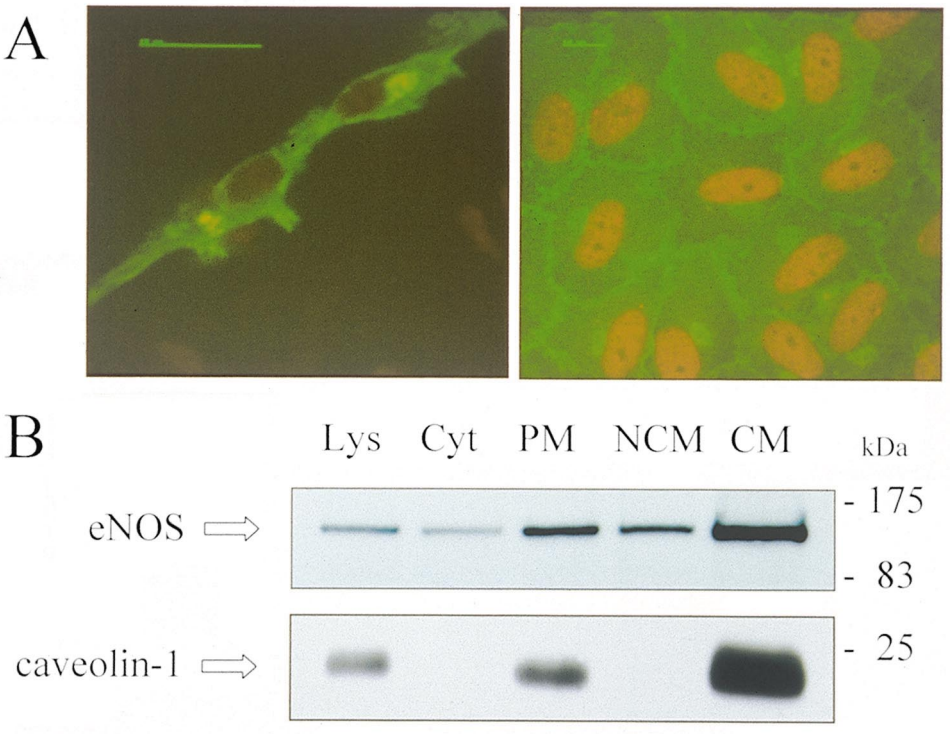

Fig. 2. Localisation of eNOS in the endothelium of native and cultured endothelial cells. (A) En face confocal microscopy of porcine coronary artery (left) and cultured human umbilical vein endothelial cells (right) labelled with a specific eNOS antibody. Note the localisation of eNOS to the plasma membrane and to the perinuclear Golgi region. In each case the bar=10 μm. (B) Western blot analysis showing the distribution of eNOS and the caveolar marker protein caveolin-1 in cell fractions prepared from native porcine aortic endothelial cells (Lys, lysate; Cyt, cytosol; PM, plasma membrane; NCM, non-caveolar membrane; Cav, caveolin-rich membrane).

One additional post-translational mechanism thought to govern the interaction between eNOS and caveolin-1 is tyrosine phosphorylation, although it is not clear at the moment whether or not one or both of the proteins must be tyrosine phosphorylated [56]. Moreover, additional proteins may be involved as preliminary evidence also suggests that additional eNOS-associated proteins are phosphorylated [57–59].

Although, there is overwhelming evidence for the existence of such a reversible protein–protein interaction, the exact mechanism in vivo is still uncertain. For example, caveolae contain relatively large concentrations of CaM and whether the stoichiometry of CaM to caveolin-1 in the vicinity of eNOS could ever reach the state at which eNOS is mostly inactive is, at the moment, unclear. Moreover, the ongoing debate regarding the competitive antagonistic roles of CaM and caveolin-1, undermines the possible relevance of the auto-inhibitory loop in the vicinity of the CaM-binding domain, in regulating eNOS function.

What happens to eNOS after dissociation from the caveolin-1 complex? This is also a point which is still largely unresolved, since, although the dissociation of eNOS from a membrane fraction (as a consequence of depalmitoylation) and its translocation to the cytosol following cell stimulation with bradykinin [60,61] has been described, this finding could not be confirmed by other groups [62]. eNOS has also been reported to translocate to a Triton X-100-insoluble/cytoskeletal cell fraction following stimulation with bradykinin [63], again this is not a universal observation [59]. In the case of bradykinin, one additional possibility has recently arisen, based on the proposed association of eNOS with the B_2 kinin receptor [64]. As this receptor is relatively rapidly desensitised and internalised via caveolae [65], it is possible that the translocation of eNOS into the cytosol represents the cycling of the enzyme bound to the B_2 receptor or a membrane component in its close vicinity.

At this stage it should be stressed that although the points discussed above are applicable to the endothelium on the whole, the endothelium of one vessel or vascular bed is not always identical with that of another. The intracellular localisation of eNOS differs in endothelial cells within the coronary system [66] as do the specific proteins expressed by the endothelium from one organ to the next [67]. There are also vessels and vascular beds in which NO plays little or no role in mediating vasodilatation and although this function is still controlled by the endothelium, the autacoids mediating this response are prostacyclin and EDHF(s).

2.3. Other modulators of eNOS activity

There is a clear disparity in the temporal relationship between agonist-induced increases in endothelial $[Ca^{2+}]_i$ and the activation of eNOS, the duration of the Ca^{2+}-response being significantly shorter than that of the subsequent NO production. Indeed, apart from changes in intracellular levels of Ca^{2+}, a number of post-translational mechanisms have been proposed to regulate eNOS activity; including the interaction of eNOS with associated proteins, or membrane phospholipids, and phosphorylation (Table 1). In addition, relatively small pH changes in the physiological range (from 6.7 to 7.4) markedly alter the activity of the eNOS derived from two different species [68]. Moreover, selective inhibition of the Na^+/H^+ exchanger induces a pronounced intracellular acidification as well as a decrease in NO production [68,69]. High concentrations of NO have been reported to inhibit NOS activity by interacting with the heme prosthetic group [70]. Additional pathways may also be implicated as the prolonged incubation of endothelial cells with NO irreversibly inhibits eNOS by a mechanism that may involve the generation of O_2 [71].

2.4.1. Associated proteins

The concept that eNOS may complex proteins which determine cellular targeting or regulate its activity is somewhat analogous to the situation described for nNOS which associates with α1-syntrophin [72], the postsynaptic density proteins (PSD-95 and PSD-93) [73] as well as a small dimer-destabilising protein [74]. Apart from calmodulin and caveolin-1, [53,58] no additional eNOS-associated proteins had been characterised until recently.

ENAP-1: ENAP-1 (endothelial NOS-associated protein-1) [63] is a tyrosine-phosphorylated, 90 kDa protein observed to interact with eNOS immunoprecipitated from cultured bovine aortic endothelial cells. The Ca^{2+}-elevating, receptor-dependent agonist bradykinin, which enhances NO production, is reported to stimulate two and one half cycles of tyrosine phosphorylation/dephosphorylation of ENAP-1 within 10 min. The functional significance of the temporal waves of tyrosine phosphorylation have not yet been elucidated [63].

Hsp90: Heat shock proteins (Hsp100, 90 70, 60 and the small Hsp/α-crystallins), so-called because they are preferentially synthesised by organisms exposed to heat or other physiological stress, are also expressed constitutively and function as molecular chaperones, able to mediate many cellular processes by influencing higher order protein structure [75]. Several signal transduction systems, especially steroid receptors, utilise an interaction with Hsp90 as an essential component of the signalling pathway and a number of signalling molecules such as G protein βγ subunits and protein kinases, including Src and Raf components of the mitogen-activated protein kinase (MAP) cascade, are also bound to Hsp90 (for review see Ref. [76]). Geldanamycin and herbimycin, commonly accepted as inhibitors of Src-family tyrosine kinases, do not inhibit the kinase activity of Src, but bind in a specific manner to Hsp90, inhibiting Src-Hsp90 heterocomplex formation and increasing Src turnover [77,78].

Hsp90 has recently been identified as an eNOS-associated protein, and its binding to the enzyme increases catalytic activity [79]. A certain amount of Hsp90 appears to complex with eNOS in unstimulated endothelial cells as immunoprecipitation of Hsp90 results in the recovery of eNOS, and vice versa. In response to cell stimulation with histamine or VEGF, Hsp90 is rapidly recruited to eNOS and exposure of endothelial cells to fluid shear stress stimulates the association of both proteins, albeit with a slower time course. In all cases the association of Hsp90 with eNOS increased NO production and was prevented by pretreatment with geldanamycin [79]. The eNOS-associated Hsp90 may also serve as a scaffolding protein, facilitating the organisation of additional associated regulatory proteins.

Given the similarity in the molecular weight of ENAP and Hsp90, it is possible that these are one and the same protein. Although Hsp90 has been described as a serine/threonine phosphorylated protein [80], co-immunoprecipitation experiments showed that genistein inhibited ligand-induced release of Hsp90 from the glucocorticoid receptor. Thus, the interaction of proteins with Hsp90 may also be regulated by a tyrosine kinase-dependent pathway [81]. Preliminary experiments also indicated that the Hsp90 which forms a complex with eNOS following the application of fluid shear stress is indeed tyrosine phos-

Table 1
Cellular and pharmacological modulators of eNOS activity in response to humoral and physical stimuli[a]

	Agonists (bradykinin, acetylcholine etc.)	Physical stimuli (shear stress, isometric contraction)
Cellular modulators		
Ca^{2+}/calmodulin	↑	↔
Caveolin-1	↓	
Hsp90	↑	↑
ENAP	?	?
↑ pH_i	↑	↑
Serine phosphorylation	↑↓	↑
Tyrosine phosphorylation	?	↑?
Pharmacological modulators		
Ro 31-8220	↑	↔
Erbstatin	↔	↓
Geldanamycin	↓	↓
Calmidazolium	↓	↔

[a] Of the pharmacological inhibitors Ro 31-8220 is a PKC inhibitor, erbstatin a tyrosine kinase inhibitor, geldanamycin an Hsp90-binding protein and calmidazolium a calmodulin antagonist. ENAP: eNOS-associated protein, ↑: increase, ↓: decrease, ↔: no effect, and ?: effect unclear.

phorylated (Unpublished observation). Additional tyrosine-phosphorylated eNOS-associated proteins (115–120 and ~150 kDa) have also been described [59], but the identity of these remains to be elucidated.

2.4.2. Phosphorylation

The role of protein kinases in the regulation of endothelial NO production is a topic of intense current investigation, since several consensus sequence sites for phosphorylation by protein kinase (PK) A, PKB (Akt), PKC and calmodulin kinase II are found in eNOS. Although eNOS was initially reported to be basally phosphorylated solely on serine residues [60,82–84] evidence that eNOS is also threonine and tyrosine phosphorylated has recently been provided [56,59]. In cultured endothelial cells, bradykinin was initially described to enhance the serine phosphorylation of eNOS, an effect which was maximal after 5 min and was maintained for at least 20 min [82]. This bradykinin-induced phosphorylation of eNOS appears to be a Ca^{2+}-dependent phenomenon and is inhibited either by a calmodulin antagonist or the removal of extracellular Ca^{2+}. A rough comparison of the time course of NO production and eNOS activation in response to bradykinin tends to suggest that at least this serine phosphorylation of eNOS may be an inactivating mechanism and fits well with the observations that PKC (which phosphorylates eNOS in vitro [83]) negatively regulates endothelial NO production [69,83,85,86]. In addition, the exposure of pulmonary artery endothelial cells to SNP, enhances the serine phosphorylation of eNOS in a PKC-dependent manner and attenuates eNOS activity [71]. Fluid shear stress also induces the serine and tyrosine phosphorylation of eNOS, together with the maintained Ca^{2+}-independent production of NO [69,87]. While definitive proof of an enhanced serine or tyrosine phosphorylation of eNOS under Ca^{2+}-free conditions is at the moment lacking, it would seem that, under these experimental conditions, the increase in phosphorylation is unlikely to be an inhibitory signal. It is most probable that the effect of phosphorylation on eNOS activity very much depends on the specific residue phosphorylated, as phosphorylation of Ser741 in the CaM-binding domain of nNOS prevents the binding of calmodulin and attenuates enzyme activity [88], while the Akt-mediated serine phosphorylation of eNOS increases enzyme activity [89].

Elaborating the functional consequences of eNOS tyrosine phosphorylation is even more complex, as this modification is only evident in primary cultured cells [56,59] and investigators using passaged endothelial cells have generally been unable to detect phosphotyrosine residues on eNOS [63,82,84]. The use of tyrosine kinase inhibitors provides only limited information on the role played by tyrosine phosphorylation in the regulation of endothelial NO production as many of these compounds directly affect Ca^{2+}-signalling processes [90,91].

2.5. Ca^{2+}-independent eNOS activation

Although classified as Ca^{2+}/CaM-dependent enzyme, a basal eNOS activity was originally reported at Ca^{2+} concentrations as low as 10 nmol/l in lysates prepared from native endothelial cells, indicating that a significant portion of the NO produced by unstimulated endothelial cells may be formed via a Ca^{2+}-independent pathway [92]. Little physiological relevance was attributed to this phenomenon, especially after the identification of a CaM-binding domain in the primary structure of eNOS. More recent thorough biochemical studies have however reinforced the original observation that eNOS may produce NO in an apparently Ca^{2+}-independent manner. While eNOS, like the neuronal isoform, requires binding of Ca^{2+}/CaM to achieve maximal NOS activity, a basal level of NO synthesis could be demonstrated even when Ca^{2+}/CaM was omitted and 0.5 mM EDTA was present in the assay solution [10]. Moreover, eNOS binds CaM so tightly that the activity of the purified enzyme is about 80% of maximum and immunoprecipitation of eNOS results in the co-precipitation of CaM, unless stringent washing procedures are used. As L-arginine binding has also been shown to stimulate heme reduction in the absence of supplementary CaM, the enzymatic synthesis of NO by eNOS appears to be partially regulated by binding of both CaM and arginine [10]. These findings for eNOS represent a significant difference from the enzymatic properties of the other NOS isoforms, and may serve to ensure the continuous production of NO, independent of fluctuations in $[Ca^{2+}]_i$. Moreover, the dependency of enzyme activity on L-arginine binding would be expected to prevent the production of O_2^- in the absence of substrate. eNOS activation in response to the application of shear stress to endothelial cells differs from that activated by receptor-dependent agonists in that it is maintained (over hours) and can be observed in the absence of extracellular Ca^{2+}, and is not inhibited by the calmodulin antagonist calmidazolium which abrogates the agonist-induced vasodilatation to acetylcholine [69,93]. A pharmacologically identical activation of eNOS can be induced by protein tyrosine phosphatase inhibitors suggesting that the tyrosine phosphorylation of eNOS or an associated regulatory protein is crucial for its Ca^{2+}-independent activation [59]. In native porcine aortic endothelial cells eNOS exists as part of a multi-molecular complex and its Ca^{2+}-independent activation seems to be linked to eNOS phosphorylation, and association of the enzyme with other proteins, as well as the redistribution of the eNOS complex to a Triton X-100-insoluble/cytoskeletal cell fraction [59]. The hypothesis that eNOS can be activated by at least two independent signalling pathways is supported by the observation that disruption of the cytoskeleton attenuated the flow-induced release of NO in native endothelial cells but failed to affect the agonist-induced production of NO [94]. In addition, RGD-containing peptides have been reported to attenuate

the flow-induced dilation of isolated coronary arterioles without affecting the response to substance P [95]. This Ca^{2+}-independent activation of eNOS is sensitive to certain kinase inhibitors notably tyrosine kinase inhibitors such as erbstatin A, and the Hsp90-binding protein, geldanamycin, but is unaffected by inhibitors of protein kinase C which potentiate acetylcholine-induced NO synthesis [69,96,96].

As a change in detergent solubility is frequently indicative of the formation of a protein complex it is tempting to speculate that fluid shear stress and tyrosine phosphatase inhibitors may alter the conformation and/or protein coupling of eNOS, facilitating its interaction with specific phospholipids, proteins and/or protein kinases which enhance/maintain its activation. Indeed, the time course of the association of Hsp90 with eNOS [79] parallels the changes in the detergent solubility of eNOS induced by stimuli which elicit its Ca^{2+}-independent activation [59]. Moreover, Hsp90-binding proteins inhibit both the shear stress-induced increase in NO production and redistribution of eNOS to a Triton X-100-insoluble (cytoskeletal) cell fraction [59]. However, since the formation of an Hsp90/eNOS complex also rapidly occurs following endothelial cell stimulation with Ca^{2+}-elevating agonists it is unlikely that the binding of Hsp90 to eNOS alone is sufficient to render its activation independent of a maintained increase in $[Ca^{2+}]_i$.

2.6. How does an endothelial cell sense physical stimuli?

In order to speculate exactly how shear stress can activate eNOS in a Ca^{2+}-independent manner it is necessary to diverse slightly and consider how an endothelial cell senses physical stimuli. The endothelial cell can be viewed as a membrane stretched over a frame composed of microtubules, intermediate filaments and actin fibres which transverse the cells and end in characteristic adhesion complexes as well as in the vicinity of caveolae. Even under non-stimulated conditions the entire endothelial cytoskeleton is maintained under tension and in response to an externally applied stimulus intracellular tension is redistributed over the cytoskeletal network. This tensegrity architecture within cells permits forces to be directly transmitted from the cell surface, through the cytoskeleton, across physically interconnecting filaments to the nucleus (for review see Ref. [97]). Thus extracellular forces are superimposed upon pre-existing forces within cells attached to the extracellular matrix at focal adhesion points, and to each other at cell–cell contacts. Generally signalling molecules are clustered around and inherent to these contact sites, as well as to plasmalemmal caveolae, so that it is conceivable that the application of a stress, which is transmitted through the entire cell by the actin cytoskeleton, activates signal transduction cascades without the need of a specific shear stress or stretch receptor. Recently, molecular connections between integrins, cytoskeletal filaments and nuclear scaffolds have been proposed to provide a pathway for signal transfer, thus raising the possibility that mechanical stimuli may be passed on to the nucleus in the absence of/or simultaneously with mechano-chemical signalling processes [98]. The localisation of eNOS, in the vicinity of the cell–cell contact [66] and within caveolin-rich membrane domains [46,47], both subcompartments of the plasma membrane in which several key signal transducing complexes are concentrated, is likely to have profound repercussions on enzyme activity as well as on its sensitivity to activation by signal transduction cascades other than those resulting in an increase in $[Ca^{2+}]_i$.

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References

[1] Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373–376.
[2] Palmer RMJ, Ferridge AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524–526.
[3] Furchgott RF. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activable inhibitory factor from bovine retractor penis is organic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: Vanhoutte PM, editor, Mechanisms of vasodilation, New York: Raven Press, 1988, pp. 401–418.
[4] Ignarro LJ, Byrns R, Buga GM, Wood RS, Chaudhuri G. Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study the endothelium-dependent and nitric oxide elicited vascular smooth muscle relaxation. J Pharmacol Exp Ther 1987;244:181–189.
[5] Chen PF, Tsai AL, Berka V, Wu KK. Mutation of Glu-361 in human endothelial nitric-oxide synthase selectively abolishes L-arginine binding without perturbing the behaviour of heme and other redox centers. J Biol Chem 1997;272:6114–6118.
[6] Sessa WC, Harrison JK, Barber CM et al. Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. J Biol Chem 1992;267:15274–15276.
[7] Ghosh DK, Wu CQ, Pitters E et al. Characterization of the inducible nitric oxide synthase oxygenase domain identifies a 48 amino acid sequence required for subunit dimerization and tetrahydrobiopterin interaction. Biochemistry 1997;36:10609–10619.
[8] Crane BR, Arvai AS, Ghosh DK et al. Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. Science 1998;279:2121–2126.
[9] Raman CS, Li H, Martásek P et al. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. Cell 1998;95:939–950.
[10] Presta A, Liu J, Sessa WC, Stuehr DJ. Substrate binding and calmodulin binding to endothelial nitric oxide synthase coregulate its enzymatic activity. Nitric Oxide 1997;1:74–87.

[11] List BM, Klosch B, Volker C et al. Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: Tetrahydrobiopterin binding kinetics and role of heme in dimerization. Biochem J 1997;323:159–165.
[12] Chen P-F, Tsai AL, Wu KK. Mutation of Glu-361 in human endothelial nitric-oxide synthase selectively abolishes L-arginine binding without perturbing the behavior of heme and other redox centers. J Biol Chem 1994;269:25062–25066.
[13] Martasek P, Liu Q, Liu J et al. Characterization of bovine endothelial nitric oxide synthase expressed in *E. coli*. Biochem Biophys Res Commun 1996;219:359–365.
[14] Abu-Soud HM, Gachhui R, Raushel FM, Stuehr DJ. The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of L-arginine and tetrahydropiopterin on its stability. J Biol Chem 1997;272:17349–17353.
[15] Abu-Soud HM, Stuehr DJ. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. Proc Natl Acad Sci USA 1993;90:10769–10772.
[16] Venema VJ, Ju H, Zou R, Ryan JW, Venema RC. Subunit interactions of endothelial nitric-oxide synthase. Comparisons to the neuronal and inducible nitric-oxide synthase isoforms. JBiolChem 1997;272:1276–1282.
[17] Siddhanta U, Presta A, Fan BC et al. Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer. J Biol Chem 1998;273:18950–18958.
[18] Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. Generation of superoxide by purified brain nitric oxide synthase. J Biol Chem 1992;267:24173–24176.
[19] Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. Circulation 1995;91:139–144.
[20] Wever RM, Vandam T, Vanrijn HJ, Degroot F, Rabelink TJ. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochem Biophys Res Commun 1997;237:340–344.
[21] Vasquezvivar J, Kalyanaraman B, Martasek P et al. Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. Proc Natl Acad Sci USA 1998;95:9220–9225.
[22] Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothleial nitric-oxide synthase. J Biol Chem 1998;273:25804–25808.
[23] Pritchard KA, Groszek L, Smalley DM et al. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. Circ Res 1995;77:510–518.
[24] Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. Circulation 1995;91:139–144.
[25] Stroes E, Kastelein J, Cosentino F et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 1998;99:41–46.
[26] Busse R, Mülsch A. Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. FEBS Lett 1990;265:133–136.
[27] Busse R, Fleming I. Regulation and functional consequences of endothelial nitric oxide formation. Ann Med 1995;27:331–340.
[28] Singer AH, Peach MJ. Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. Hypertension 1982;4:II-19–II-25.
[29] Lückhoff A, Pohl U, Mülsch A, Busse R. Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. Br J Pharmacol 1988;95:189–196.
[30] Nishida K, Harrison DG, Navas JP et al. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. J Clin Invest 1992;90:2092–2096.
[31] Bredt DS, Huang PM, Glatt CE et al. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 1991;351:714–718.
[32] Marsden PA, Schappert KT, Chen HS et al. Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett 1992;307:287–293.
[33] Venema RC, Sayegh HS, Kent JD, Harrison DG. Identification, characterization, and comparison of the calmodulin-binding domains of the endothelial and inducible nitric oxide synthases. J Biol Chem 1996;271:6435–6440.
[34] Venema RC, Sayegh HS, Arnal J-F, Harrison DG. Role of enzyme calmodulin-binding domain in membrane association and phospholipid inhibition of endothelial nitric oxide synthase. J Biol Chem 1995;270:14705–14711.
[35] Matsubara M, Titani K, Taniguchi H. Interaction of calmodulin-binding domain peptides of nitric oxide synthase with membrane phospholipids: regulation by protein phosphorylation and Ca^{2+}–calmodulin. Biochemistry 1996;35:14651–14658.
[36] Gachhui R, Abu-Soud HM, Ghoshà DK et al. Neuronal nitric oxide synthase interaction with calmodulin–troponin C chimeras. J Biol Chem 1998;273:5451–5454.
[37] Ghosh S, Gachhui R, Crooks C et al. Interaction between caveolin-1 and the reductase domain of endothelial nitric-oxide synthase. Consequences for catalysis. J Biol Chem 1998;273:22267–22271.
[38] Salerno JC, Harris DE, Irizarry K et al. An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. J Biol Chem 1997;272:29769–29777.
[39] Lisanti MP, Scherer PE, Tang Z, Sargiacomo M. Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. Trends Cell Biol 1994;4:231–235.
[40] Rothberg KG, Heuser JE, Donzell WC et al. Caveolin, a protein component of caveolae membrane coats. Cell 1992;68:673–682.
[41] Okamoto T, Schlegel A, Scherer PE, Lisanti MP. Caveolins, a family of scaffolding proteins for organizing 'preassembled signaling complexes at the plasma membrane'. J Biol Chem 1998;273:5419–5422.
[42] Sessa WC, García-Cardena G, Liu J et al. The Golgi association of endothelial nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. J Biol Chem 1995;270:17641–17644.
[43] O'Brien AJ, Young HM, Povey JM, Furness JB. Nitric oxide synthase is localized predominantly in the Golgi apparatus and cytoplasmic vesicles of vascular endothelial cells. Histochemistry 1995;103:221–225.
[44] Liu J, Hughes TE, Sessa WC. The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. J Cell Biol 1997;137:1525–1535.
[45] Hecker M, Mülsch A, Bassenge E, Förstermann U, Busse R. Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications. Biochem J 1994;299:247–252.
[46] Shaul PW, Smart EJ, Robinson LJ et al. Acylation targets endothelial nitric oxide synthase to plasmalemmal caveolae. J Biol Chem 1996;271:6518–6522.
[47] Feron O, Belhassen L, Kobzik L et al. Endothelial nitric oxide synthase targeting to caveolae: Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. J Biol Chem 1996;271:22810–22814.
[48] Liu J, García-Cardena G, Sessa WC. Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. Biochemistry 1996;35:1.
[49] García-Cardena G, Oh P, Liu J, Schnitzer JE, Sessa WC. Targeting of nitric oxide synthase to endothelial caveolae via palmitoylation: implications for nitric oxide signaling. Proc Natl Acad Sci USA 1996;93:6448–6453.
[50] Bauersachs J, Fleming I, Scholz D, Popp R, Busse R. Endothelium-derived hyperpolarizing factor but not nitric oxide is reversibly inhibited by brefeldin A. Hypertension 1997;30:1598–1605.

[51] Couet J, Li S, Okamoto T, Ikezu T, Lisanti MP. Identification of peptide and protein ligands for the caveolin-scaffolding domain Implications for the interaction of caveolin with caveolae-associated proteins. J Biol Chem 1997;272:6525–6533.
[52] Michel JB, Feron O, Sase K, Prabhakar P, Michel T. Caveolin versus calmodulin: Counterbalancing allosteric modulators of endothelial nitric oxide synthase. J Biol Chem 1997;272:25907–25912.
[53] García-Cardena G, Martasek P, Masters BS et al. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin: Functional significance of the NOS caveolin binding domain in vivo. J Biol Chem 1997;272:25437–25440.
[54] Ju H, Zou R, Venema VJ, Venema RC. Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. J Biol Chem 1997;272:18522–18525.
[55] Michel JB, Feron O, Sacks D, Michel T. Reciprocal regulation of endothelial nitric-oxide synthase by Ca^{2+}-calmodulin and caveolin. J Biol Chem 1997;272:15583–15586.
[56] García-Cardena G, Fan G, Stern DF, Liu J, Sessa WC. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. J Biol Chem 1996;271:27237–27240.
[57] Fleming I, Fisslthaler B, Busse R. The endothelial NO synthase exists in situ as a protein complex the constituents of which are altered following endothelial cell stimulation with a tyrosine phosphatase inhibitor [abstract]. Nitric Oxide 1998;2:91.
[58] Venema VJ, Zou R, Ju H, Marrero MB, Venema RC. Caveolin-1 detergent solubility and association with endothelial nitric oxide synthase is modulated by tyrosine phosphorylation. Biochem Biophys Res Commun 1997;236:155–161.
[59] Fleming I, Bauersachs J, Fisslthaler B, Busse R. Calcium-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. Circ Res 1998;81:686–695.
[60] Robinson LJ, Busconi L, Michel T. Agonist-modulated palmitoylation of endothelial nitric oxide synthase. J Biol Chem 1995;270:995–998.
[61] Prabhakar P, Thatte HS, Goetz RM et al. Receptor-regulated translocation of endothelial nitric oxide synthase. J Biol Chem 1998;273:27383–27388.
[62] Liu J, García-Cardena G, Sessa WC. Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. Biochemistry 1995;34:12333–12340.
[63] Venema VJ, Marrero MB, Venema RC. Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. Biochem Biophys Res Commun 1996;226:703–710.
[64] Ju H, Venema VJ, Marrero MB, Venema RC. Inhibitory interactions of the bradykinin B_2 receptor with endothelial nitric oxide synthase. J Biol Chem 1998;273:24025–24029.
[65] Benzing T, Fleming I, Blaukat A, Müller-Esterl W, Busse R. The ACE inhibitor ramiprilat prevents the sequestration of the B_2 kinin receptor within the plasma membrane of native endothelial cells. Circulation 1999;99:2034–2040.
[66] Andries LJ, Brutsaert DL, Sys SU. Nonuniformity of endothelial constitutive nitric oxide synthase distribution in cardiac endothelium. Circ Res 1998;82:195–203.
[67] Rajotte D, Arap W, Hagedorn M et al. Molecular heterogeneity of the vascular endothelium revealed by in vitro phage display. J Clin Invest 1998;102:430–437.
[68] Fleming I, Hecker M, Busse R. Intracellular alkalinization induced by bradykinin sustains activation of the constitutive nitric oxide synthase in endothelial cells. Circ Res 1994;74:1220–1226.
[69] Ayajiki K, Kindermann M, Hecker M, Fleming I, Busse R. Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. Circ Res 1996;78:750–758.
[70] Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ. Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. J Biol Chem 1994;269:21644–21649.
[71] Sheehy AM, Burson MA, Black SM. Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: a role for superoxide. Am J Physiol 1998;247:L833–L841.
[72] Chao DS, Gorospe JRM, Brenman JE et al. Selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy. J Exp Med 1996;184:609–618.
[73] Brenman JE, Chao DS, Gee SH et al. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α1-syntrophin mediated by PDZ domains. Cell 1996;84:757–767.
[74] Jaffrey SR, Snyder SH. PIN: An associated protein inhibitor of neuronal nitric oxide synthase. Science 1996;274:774–777.
[75] Liang P, Macrae TH. Molecular chaperones and the cytoskeleton. J Cell Sci 1997;110:1431–1440.
[76] Pratt WB. The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptor signaling via MAP kinase. Annu Rev Pharmacol Toxicol 1997;37:297–326.
[77] Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60$^{v\text{-}src}$ heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci USA 1994;91:8324–8328.
[78] Uehara Y, Murakami Y, Sugimoto Y, Mizuno S. Mechanism of reversion of Rous sarcoma virus transformation by herbimycin A: reduction of total phosphotyrosine levels due to reduced kinase activity and increased turnover of p60^{vsrc1}. Cancer Res 1989;49:780–785.
[79] García-Cardena G, Fan R, Shah V et al. Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature 1998;292:821–824.
[80] Mimnaugh EG, Worland PJ, Whitesell L, Neckers LM. Possible role for serine/threonine phosphorylation in the regulation of the heteroprotein complex between the hsp90 stress protein and the pp60v-src tyrosine kinase. J Biol Chem 1995;270:28654–28659.
[81] Gradin K, Whitelaw ML, Toftgard R, Poellinger L, Berghard A. A tyrosine kinase-dependent pathway regulates ligand-dependent activation of the dioxin receptor in human keratinocytes. J Biol Chem 1998;269:23800–23807.
[82] Michel T, Li GK, Busconi L. Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. Proc Natl Acad Sci USA 1993;90:6252–6256.
[83] Hirata K, Kuroda R, Sakoda T et al. Inhibition of endothelial nitric oxide synthase activity by protein kinase C. Hypertension 1995;25:180–185.
[84] Corson MA, James NL, Latta SE et al. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ Res 1996;79:984–991.
[85] Tsukahara H, Gordienko DV, Goligorsky M. Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. Biochem Biophys Res Commun 1993;193:722–729.
[86] Davada RK, Chandler LJ, Guzman NJ. Protein kinase C modulates receptor-independent activation of endothelial nitric oxide synthase. Eur J Pharmacol 1994;266:237–244.
[87] Fleming I, Fisslthaler B, Busse R. Shear stress-induced phosphorylation of the endothelial NO synthase [abstract]. Nitric Oxide 1998;2:76.
[88] Zoche M, Beyermann M, Koch KW. Introduction of a phosphate at serine(741) of the calmodulin-binding domain of the neuronal nitric oxide synthase (NOS-I) prevents binding of calmodulin. Biol Chem 1997;378:851–857.
[89] Dimmeler S, Fisslthaler B, Fleming I et al. Shear stress stimulates the protein kinase Akt-involvement in regulation of the endothelial nitric oxide synthase [abstract]. Circulation 1998;98:I-312.
[90] Fleming I, Fisslthaler B, Busse R. Interdependence of calcium signaling and protein tyrosine phosphorylation in human endothelial cells. J Biol Chem 1996;271:11009–11015.

[91] Fleming I, Busse R. Tyrosine phosphorylation and bradykinin-induced signaling in endothelial cells. Am J Cardiol 1997;80:102A–109A.
[92] Mülsch A, Bassenge E, Busse R. Nitric oxide synthesis in endothelial cytosol: evidence for a calcium-dependent and a calcium-independent mechanism. Naunyn Schmiedeberg's Arch Pharmacol 1989;340:767–770.
[93] Kuchan MJ, Frangos JA. Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. Am J Physiol 1994;266:C628–C636.
[94] Hutcheson IR, Griffith TM. Mechanotransduction through the endothelial cytoskeleton: mediation of flow- but not agonist-induced EDRF release. Br J Pharmacol 1996;118:720–726.
[95] Muller JM, Chilian WM, Davis MJ. Integrin signaling transduces shear stress-dependent vasodilation of coronary arterioles. Circ Res 1997;80:320–326.
[96] Fleming I, Bauersachs J, Busse R. Calcium-dependent and -independent activation of the endothelial NO synthase. J Vasc Res 1997;34:165–174.
[97] Ingber DE. Tensegrity: The architectural basis of cellular mechanotransduction. Annu Rev Physiol 1997;59:575–599.
[98] Maniotis AJ, Chen CS, Ingber DE. Demonstration of mechanical connections between integrins cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci USA 1997;94:849–854.