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## eNOS activation and NO function: Structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity

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### Abstract

Rather than being a constitutive enzyme as was first suggested, endothelial nitric oxide synthase (eNOS) is dynamically regulated at the transcriptional, posttranscriptional, and posttranslational levels. This review will focus on how changes in eNOS function are conferred by various posttranslational modifications. The latest knowledge regarding eNOS targeting to the plasma membrane will be discussed as the role of protein phosphorylation as a modulator of catalytic activity. Furthermore, new data are presented that provide novel insights into how disruption of the eNOS dimer prevents eNOS uncoupling and the production of superoxide under conditions of elevated oxidative stress and identifies a novel regulatory region we have termed the ‘flexible arm’.

### Introduction

Nitric oxide (NO) is synthesized by a family of enzymes called NO synthases (NOS). There are three mammalian NOS isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). They share 50–60% homology at the amino acid level and have an N-terminal oxygenase domain with heme-, L-arginine-, tetrahydrobiopterin (BH<sub>4</sub>)-binding domains, a central calmodulin (CaM)-binding region, and a C-terminal reductase domain with NADPH, FAD, and FMN binding sites (Stuehr 1997). Under physiological conditions, the dominant NOS isoform in the vasculature is eNOS, which rather than being a constitutive enzyme as was first suggested, is dynamically regulated at the transcriptional, posttranscriptional, and posttranslational levels. This review will focus on the posttranslational modifications that regulate eNOS activity and will conclude with the identification of a new structural component of eNOS that regulates enzyme function under conditions of oxidative stress.

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#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Membrane targeting of eNOS

In contrast to the other NOS isoforms, eNOS contains a myristoyl group that is covalently attached to the glycine residue found at the second amino acid in the N-terminal region of the protein (Sessa *et al.* 1993). The co-translational addition of this myristoyl group enables eNOS to localize to cellular membranes and also serves as a mechanism to ensure that eNOS is in close proximity with other factors that regulate its function. Mutational studies have shown that the myristoyl group is an absolute requirement for membrane localization and maximum eNOS activity (Sakoda *et al.* 1995). In cells, the loss of *N*-myristoylation confers a cytosolic location and reduced eNOS activity, but in isolated activity assays with maximal cofactors, eNOS activity is equal to the wild-type enzyme (Sessa *et al.* 1993). It is thought that myristoylation targets eNOS to the Golgi complex (Sessa *et al.* 1995), and although the myristoyl deficient eNOS can metabolize L-arginine and generate NO, it exhibits reduced coupling to external stimuli such as calcium ionophores (Sessa *et al.* 1995). eNOS is also subject to posttranslational palmitoylation on two cysteine residues (Cys-15 and Cys-26) within the oxygenase domain of the enzyme. This modification is reversible and requires the prior *N*-myristoylation and membrane anchoring of eNOS. It is believed that palmitoylation stabilizes the association of eNOS with the membrane and may also be required for correct localization of the enzyme within specialized lipid domains (Liu *et al.* 1995). The role of palmitoylation in eNOS activation is controversial. Reports have indicated that agonist-induced activation of eNOS is associated with depalmitoylation and translocation from membranes (Yeh *et al.* 1999). However, this study is contradicted by a similar report (Liu *et al.* 1995). Mutation of the palmitoylation sites has been shown to decrease agonist-stimulated eNOS activity, but to a lesser extent than for the myristoyl-deficient mutant (Shaul *et al.* 1996). The palmitoyl-deficient mutant also has an altered subcellular distribution in that it is virtually absent from the plasma membrane (Robinson & Michel 1995, Garcia-Cardena *et al.* 1996b). As with myristoyl-deficient eNOS, it should be noted that the palmitoyl-deficient enzyme is not catalytically inferior to the wild-type enzyme as the purified enzymes are kinetically identical (Liu *et al.* 1996). One particular site in the plasma membrane that is of great importance for eNOS function is the caveolae. Caveolae are specialized invaginations of the plasma membrane that are present in most cell types but are particularly enriched within the membranes of endothelial cells (EC), adipocytes, fibroblasts, and smooth muscle cells. It is thought that caveolae are crucial sites for the origination and integration of many signal transduction pathways (Anderson 1993). Studies indicate that, in the plasma membrane, eNOS is located in caveolae (Shaul *et al.* 1996) and that this location is important for eNOS activity. The localization of eNOS within the caveolae renders the enzyme inactive due to the interaction of eNOS with caveolin-1 (Ju *et al.* 1997). This interaction requires that eNOS be both myristoylated and palmitoylated, at least *in vivo* (Sessa *et al.* 1993). The mechanism by which caveolin-1 reduces eNOS activity is by preventing CaM binding when calcium levels are low (Ju *et al.* 1997, Michel *et al.* 1997) and caveolin-1 over-expression reduces basal eNOS activity. The interaction sequence for caveolin-1 in human eNOS has been located to amino acids 350–358, and when this region is mutated, eNOS activity is no longer affected by the level of caveolin-1 expression (Garcia-Cardena *et al.* 1997). However, it should be noted that there is also evidence for additional caveolin-1 binding sites, particularly interactions within the electron transport domain of eNOS, but the exact residues involved are yet to be described (Ghosh *et al.* 1998). Elevation of intracellular calcium levels or exposure of EC to shear stress causes caveolin-1 to become displaced by the active calcium/CaM complex. This event may also coincide with depalmitoylation (Robinson & Michel 1995) and translocation of eNOS to the cytosol (Michel *et al.* 1993). However, as mentioned previously, the depalmitoylation/translocation hypothesis has not been confirmed by all groups (Liu *et al.* 1995), and although the translocation of eNOS from membrane to cytosol has been demonstrated in a number of studies, its biological significance is not yet clear. It is also worth noting that eNOS has also

been found within the Golgi complex both *in vitro* and *in vivo* (Sessa *et al.* 1995, Andries *et al.* 1998). Artificially restricting eNOS expression to the Golgi reveals that eNOS expression on the cytoplasmic leaflet of the *cis* Golgi membrane results in an enzyme that is active but exhibits blunted capacity to generate NO versus the plasma membrane pool of eNOS (Fulton *et al.* 2004). Interestingly, targeting eNOS to regions of *trans* Golgi resulted in dramatically less activity suggesting that distinct regions of the Golgi are supportive or inhibitory to eNOS activity (Jagnandan *et al.* 2005). While the exact function of the Golgi eNOS is unclear at the present time, acute modulation of cholesterol levels dramatically impacts the activity of eNOS at the plasma membrane but does not alter the output of the Golgi eNOS (Zhang *et al.* 2006). It may also be that the Golgi is the location for re-palmitoylation after agonist-induced activation and depalmitoylation when the enzyme is recycled to the plasma membrane via the Golgi. Indeed, the discovery of at least five enzymes including DHHC-21 that are localized to the Golgi and promote eNOS palmitoylation provide strong evidence in support of this hypothesis (Fernandez-Hernando *et al.* 2006). Interestingly, recent data have shown that, at least under conditions of oxidative stress, eNOS trafficking to the plasma membrane is positively regulated by caveolin-1 (Tian *et al.* 2010). This enrichment of eNOS in caveolae is called the compartmentation effect (Garcia-Cardena *et al.* 1996b, Shaul *et al.* 1996) while the inhibitory effect of caveolin-1 is known as the clamp effect (Garcia-Cardena *et al.* 1996a, Michel *et al.* 1997, Feron *et al.* 1998). Thus, changes in the expression of caveolin-1 can alter the balance of eNOS regulation and consequently alter NO generation. It has also been demonstrated that eNOS can interact with the 90 kDa heat-shock protein 90 (Hsp90). Hsp90 is a molecular chaperone that can modulate protein folding and activity. Hsp90 appears to increase eNOS activity by facilitating the CaM-induced displacement of caveolin-1 from eNOS (Gratton *et al.* 2000), which can be inhibited with the Hsp90 inhibitor, geldanamycin (Garcia-Cardena *et al.* 1998).

## Phosphorylation of eNOS

eNOS can be regulated by multiple phosphorylation sites at tyrosine (Y), serine (S), and threonine (T) residues. The Y sites so far identified localize to Y81 and Y567, the S sites localize to S114, S615, S633, and S1177, and the T site localizes to T495 (using the human sequence nomenclature). The role of each phosphorylation site in regulating eNOS activity will be discussed below.

### Tyrosine phosphorylation and eNOS

Both eNOS and nNOS differ from iNOS in their primary dependence on the presence of the calcium/CaM. Thus, eNOS activity can be acutely regulated by increases in the concentration of intracellular calcium that occurs secondary to agonists such as bradykinin (Gosink & Forsberg 1993), estradiol (Goetz *et al.* 1999), and vascular endothelial growth factor (VEGF) (Papapetropoulos *et al.* 1997). Fluid shear stress (FSS) can also activate eNOS in a different manner. The initial response of eNOS to FSS is calcium-dependent (Fleming & Busse 1995, Corson *et al.* 1996), but the long-term response appears to be less dependent on changes in intracellular calcium levels. This long-term activation is reduced by tyrosine kinase inhibitors (Ayajiki *et al.* 1996, Corson *et al.* 1996) and recent data have shown that the phosphorylation of tyrosine (Y) residues regulate the ability of eNOS to produce NO (Fulton *et al.* 2005, 2008). The respective residues have been identified as Y81 (Fulton *et al.* 2005) and Y657 (Fisslthaler *et al.* 2008). Interestingly these residues exert opposing effects on eNOS activity. Y81 is phosphorylated by pp60<sup>src</sup> and activates the enzyme (Fulton *et al.* 2005, 2008) while Y567 is phosphorylated by proline-rich tyrosine kinase 2 (PYK2) and appears to attenuate eNOS activity (Fisslthaler *et al.* 2008). Both pp60<sup>src</sup> and PYK2 are activated by shear, so it is not readily apparent why it is necessary to have opposing phosphorylation events that activate and inhibit eNOS activity. One possibility is that PYK2 activation may limit the generation of peroxynitrite from eNOS in

response to sustained increases in flow (Fisslthaler *et al.* 2008). It has been noted that tyrosine phosphorylation appears to be most prominent in primary EC and may be lost when cells are cultured (Garcia-Cardena *et al.* 1996a, Fleming *et al.* 1998). This may account for the studies that have found no eNOS tyrosine phosphorylation (Corson *et al.* 1996, Venema *et al.* 1996, 1997, Dimmeler *et al.* 1999).

### Serine and threonine phosphorylation and eNOS

Considerably more is known about the serine phosphorylation of eNOS. eNOS becomes rapidly serine phosphorylated when EC are exposed to FSS leading to phosphorylation of S1177 (human enzyme) that is mediated by AKT1 (Fulton *et al.* 1999, Gallis *et al.* 1999) and other kinases such as protein kinase A (PKA) and AMPK (Chen *et al.* 1999, Michell *et al.* 2001). Increases in intracellular calcium levels activate CaM that in turn activates CaM kinase II, which also phosphorylates S1177 (Fleming *et al.* 2001). AKT1 signaling is thus important for both agonist and FSS activation of eNOS (Dimmeler *et al.* 1999). Although the AKT1-mediated activation of eNOS by FSS has been reported to be independent of changes in intracellular calcium (Dimmeler *et al.* 1999), it is more likely that the phosphorylation of S1177 renders the enzyme more responsive to much lower levels of calcium (McCabe *et al.* 2000). FSS has also been shown to stimulate the phosphorylation of eNOS at S633 within an internal autoinhibitory (AI) loop (see section The AL loop of eNOS) and S1177 by a PKA-dependent mechanism (Boo *et al.* 2003). Mimicking this effect through increased expression of the active catalytic subunit of PKA also leads to the dephosphorylation of T495 (Boo *et al.* 2003). Using eNOS point mutants where S633 was mutated to either A (to prevent phosphorylation) or D (to mimic the charge of the phosphate group), it was then shown that NO production in S633D-expressing HEK-293 or bovine aortic EC was higher than that obtained from either wild-type eNOS or the S633A eNOS mutant (Boo *et al.* 2003). Importantly, S633D expressing cells were also able to generate NO in the presence of the calcium chelator, BAPTA-AM (Boo *et al.* 2003). *In vitro* analysis of mutants of eNOS containing phosphomimics (aspartic acid mutations) at S615 or S1177 has also demonstrated that phosphorylation of these sites significantly lowers the EC<sub>50</sub> for both CaM binding and enzyme activation from the wild-type values of 180±2 and 397±23 nM to 109±2 and 258±11 nM respectively (Tran *et al.* 2008) for S615 and to 109±2 and 258±11 nM respectively for S1177 (Tran *et al.* 2009). Although introducing aspartic acid at both S617 and S1177 did not enhance the V<sub>max</sub> of eNOS, the double phosphorylation mutant protein does exhibit a further reduction in the EC<sub>50</sub> for both CaM binding and enzyme activation to 77±2 and 130±5 nM. Similarly, in cultured human aortic EC, the introduction of an alanine at aa615 (S615A, phospho-null) into the phosphomimic S1177D mutant eNOS significantly attenuates NO synthesis at resting calcium states (Ritchie *et al.* 2010). Conversely, mimicking the phosphorylation of S615 by introducing an aspartic acid (S615D) enhances NO generation (Ritchie *et al.* 2010). These data suggest that there is cooperation between AKT1-mediated phosphorylation of S615 and S1177 to enhance NO generation from eNOS at resting calcium concentrations.

Other factors such as bradykinin also stimulate NO synthesis by increasing the phosphorylation of S1177. However, many of these agents also induce phosphorylation of eNOS at S615 and S633 while stimulating the dephosphorylation of eNOS at T495 (Harris *et al.* 2001). eNOS can be phosphorylated at T495 by PKC and AMPK and this is thought to be associated with eNOS inhibition (Chen *et al.* 1999, Michell *et al.* 2001). In fact, the paradigm in the literature is that there is a yin–yang relationship between T495 and S1177 with phosphorylation of one being associated with dephosphorylation of the other. However, this is not always the case as a recent study indicated that increases in S1177 could maintain NO signaling despite enhanced T495 phosphorylation (Li *et al.* 2007, Hsu *et al.* 2010). eNOS can also be phosphorylated at S114 and this is thought to produce a decrease in

enzyme activity (Li *et al.* 2007). More recently, the MAPK Erk1/2 was shown to be the kinase responsible for S114 phosphorylation and this alters the ability of eNOS to bind the prolyl isomerase Pin1, which negatively impacts eNOS activity (Ruan *et al.* 2011). The regulation of eNOS phosphorylation also involves the activity of the protein phosphatase 2A (PP2A; Greif *et al.* 2002, Church & Fulton 2006), PP1 (Schmitt *et al.* 2009), and calcineurin (PP2B; Kou *et al.* 2002), but the contribution of individual phosphatases to the site-specific dephosphorylation of eNOS remains largely unexplored.

## The AI loop of eNOS

Within the three NOS isoforms, there are substantial differences in the rate of electron flow between reductase and oxygenase domains with eNOS having the lowest rate. Studies have shown that this is due to sequences located within the reductase domain since chimeras in which the eNOS oxygenase domain was fused to the iNOS or nNOS reductase domain exhibited higher electron flow than wild-type eNOS while the opposite was true with chimeras in which the iNOS or nNOS oxygenase domains were fused to the eNOS reductase domain (Nishida & Ortiz de Montellano 1999). By aligning the primary sequences of the NOS reductase domain, a 43–45 amino acid insert (at residue 595 of eNOS and 835 of nNOS) was identified that was absent in iNOS (Nishida & Ortiz de Montellano 1999). This region was termed the AI element (Nishida & Ortiz de Montellano 1999) as its deletion from either nNOS or eNOS leads to an increase in CaM-dependent NO synthesis in both enzymes such that activity approached that found in iNOS (Nishida & Ortiz de Montellano 1999, Nishida & de Montellano 2001), although the Ca<sup>2+</sup> independence found in iNOS was not observed. Synthetic peptides based on the 45 amino acid insert of eNOS <sup>601</sup>SSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRK<sup>633</sup> were found to prevent the binding of CaM and the activation of both eNOS and nNOS (Salerno *et al.* 1997). Other shorter peptides also exhibited an inhibitory effect leading the authors to conclude that the inhibitory peptide requires the RRKRK motif (Salerno *et al.* 1997). They also hypothesized that there was another region in the peptide that may interact with EERKSYKVERF and EQHKSYKIRF sequences that are present as insertions within the reductase domains of both eNOS and nNOS (Salerno *et al.* 1997). An additional AI region (aa1165–1178) was found in the reductase of eNOS but absent in iNOS. Disruption of this region alone slightly improves eNOS calcium sensitivity, but deletion of both the 45aa and the 14aa domains results in an eNOS enzyme that is constitutively active and profoundly calcium insensitive (Chen & Wu 2003, Church & Fulton 2006). It is also noteworthy that the major eNOS phosphorylation sites (S615, S633, and S1177) lie within these two domains. Indeed the generation of an eNOS truncation mutant resulting from the deletion of the last 27 amino acids of eNOS (1177–1205; the C-terminal 27 amino acids) produced an enzyme that had an EC<sub>50</sub> for calcium that was five times less than the wild-type eNOS and a significant increase in catalytic activity (Lane & Gross 2002), which is very similar to the enzyme kinetics of the S1177D mutant eNOS which mimics the phosphorylation of S1177 (McCabe *et al.* 2000). Furthermore, it has been postulated that the AI element interacts with the FMN-binding domain to reduce electron flow (Lane & Gross 2002). However, as a full-length eNOS (or nNOS) protein has not as yet been crystallized, this possibility remains unproven. In addition, whether there are other ways to regulate the release of the AI element remains unknown.

## BH<sub>4</sub> and eNOS

BH<sub>4</sub> is a cofactor essential for the catalytic activity of all three NOS isoforms (Kwon *et al.* 1989, Tayeh & Marletta 1989, Mayer & Hemmens 1997, Gross *et al.* 2000). Studies indicate that cellular BH<sub>4</sub> levels have important consequences for the structure of NOS enzymes. BH<sub>4</sub> binding causes NOS to shift its heme iron to a high-spin state, increases L-arginine

binding, and, at least in some NOS isoforms, stabilizes the active dimeric form of the enzyme (Gross *et al.* 2000). Suboptimal concentrations of BH<sub>4</sub> reduce the generation of NO and favor NOS ‘uncoupling’ leading to NOS-mediated reduction of oxygen and the formation of superoxide anions and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at the expense of NO. Enhanced BH<sub>4</sub> degradation in arteries exposed to oxidative stress likely contribute to the pathogenesis of endothelial dysfunction in hypertension, hypercholesterolemia, diabetes, smoking, and ischemia–reperfusion (Milstien & Katusic 1999, Heitzer *et al.* 2000, Shinozaki *et al.* 2000, Tiefenbacher *et al.* 2000, Baker *et al.* 2001, Katusic 2001). Supporting this hypothesis, studies have shown that endothelial function can be normalized by BH<sub>4</sub> supplementation in experimental animal models of insulin resistance and hypercholesterolemia (Laursen *et al.* 1997, Shinozaki *et al.* 1999, 2000, Tiefenbacher *et al.* 2000). BH<sub>4</sub> is a potent reducing agent and exhibits antioxidant activity against both superoxide anions and hydroxyl radicals (Kojima *et al.* 1995). Oxidative stress can lead to excessive oxidation and depletion of BH<sub>4</sub> and previous studies indicate that BH<sub>4</sub> is a molecular target for oxidative stress and can cause ‘uncoupling’ of eNOS (Shinozaki *et al.* 2000, Tiefenbacher *et al.* 2000, Katusic 2001). Consistent with these findings, vitamin C has been shown to stimulate NOS activity via chemical stabilization of BH<sub>4</sub> in cultured human umbilical vein EC (Huang *et al.* 2000, Heller *et al.* 2001). This effect of vitamin C appears to be independent of the chemical antagonism between vitamin C and superoxide anions (Baker *et al.* 2001).

The exact redox mechanism by which BH<sub>4</sub> participates in the biosynthesis of NO is still not completely understood (Gross *et al.* 2000). However, accumulated evidence indicates that optimal concentrations of BH<sub>4</sub> are fundamentally important for the normal function of eNOS in EC. Information gained from the work of Klatt *et al.* (1995) indicates that BH<sub>4</sub> is intimately involved in maintaining nNOS dimers. In contrast, the work of Rodríguez-Crespo & Ortiz de Montellano (1996) suggests that BH<sub>4</sub> may not be required for eNOS dimerization. However, the study of N-terminal deletions in bovine eNOS revealed that the deletion of 91 or 105 amino acids reduced the ability of the enzyme to dimerize (Rodríguez-Crespo *et al.* 1997). This suggests that some of the protein residues involved in dimer formation may be located in amino acids 52–105 of eNOS. The crystal structure for the heme domain of eNOS has been solved (Raman *et al.* 1998) and reveals that zinc ion is tetrahedrally coordinated by two pairs of symmetry-related cysteine residues (corresponding to C94 and C99 from each monomer) predicted to be located at the eNOS dimer interface (Fig. 1).

## Regulation of eNOS by dimerization

The active form of eNOS enzyme exists as two identical subunits that form a head to tail homodimer. However, the dimeric interface of the two eNOS monomers has not been entirely elucidated. It has been previously shown that cysteines 94 and 99 of eNOS form a zinc tetra-coordinated (ZnS<sub>4</sub>) cluster between each subunit (Fig. 1A). Zinc bound to the tetrathiolate cluster has also been shown to stabilize the dimer interface on the N-terminal region of eNOS (Raman *et al.* 1998, Hemmens *et al.* 2000). However, the molecular mechanisms by which the ZnS<sub>4</sub> cluster regulates the catalytic activity of eNOS are poorly understood. Comparative analysis of bacterial NOS and cytochrome P450 structures, which are the closest homologues to NOS, reveal the ZnS<sub>4</sub> cluster to be a unique redox-sensitive structural feature of eNOS (Fig. 1A). The bacterial NOS from *Bacillus subtilis* (bsNOS; Wang *et al.* 2007) also does not have a ZnS<sub>4</sub> cluster (Fig. 2A). Interestingly, it has been recently shown that cytochrome P450 undergoes a structural rearrangement in the catalytic domain (Scott *et al.* 2003, Savino *et al.* 2009). Previous X-ray structures of P450 have been solved in a closed conformation, but recent studies have identified a structure with an open channel allowing substrate access (Scott *et al.* 2003, Savino *et al.* 2009; Fig. 2B).

Interestingly, within this structure, located between amino acids 100–109 within Helix B' (Honma *et al.* 2005), lies a flexible region of P450 (Fig. 2B). The N-terminal region of eNOS is also poorly described in the available crystal structures for eNOS, and more specifically, the N-terminal amino acids 105–125 is not present in the X-ray structure of human eNOS. This region, which we have termed the flexible arm, links the ZnS<sub>4</sub> cluster with the rest of the protein structure. This flexible region appears to be conserved from mammalian P450s to NOS (Table 1) and it is possible that this sequence may be of importance in regulating the catalytic activity of eNOS.

Regulation of NO production during conditions of oxidative stress is very important for cell survival. Previous studies have shown that increased oxidative stress is often associated with decreased NO levels (Wiseman *et al.* 2007, Yada *et al.* 2007, Gracia-Sancho *et al.* 2008). NO production, together with increased superoxide generation, leads to elevated peroxynitrite (ONOO<sup>-</sup>) formation (Beckman *et al.* 1990). ONOO<sup>-</sup> is a powerful oxidant and nitrative agent that has been shown to corrupt protein function, mediate cellular dysfunction, and induce apoptosis (Poderoso 2009). It has been shown that the ZnS<sub>4</sub> cluster is highly sensitive to oxidants such as ONOO<sup>-</sup>, NO, and H<sub>2</sub>O<sub>2</sub> (Zou *et al.* 2002, Ravi *et al.* 2004, Tummala *et al.* 2008), and the oxidation of the ZnS<sub>4</sub> cluster results in monomerization of eNOS and inhibition of catalytic activity. Thus, the inhibition of NO synthesis through dimer dissociation could play a protective role within EC experiencing increased superoxide generation. Another consideration is that, even after dimer dissociation, the monomeric eNOS retains its heme and can continue to interact with reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> (Porasuphatana *et al.* 2001, Woodward *et al.* 2009). This would result in the formation of reactive iron–oxygen compounds on the heme. Formation of highly oxidative heme states, in turn, could induce oxidation of surrounding proteins with further self-amplification of oxidative damage.

It is worthwhile noting that the effect of oxidants such as H<sub>2</sub>O<sub>2</sub> on eNOS function is complex. It is now apparent that ROS such as the superoxide anion and H<sub>2</sub>O<sub>2</sub> can act as second messenger molecules (Griendling *et al.* 2000) altering the function of specific proteins; although in most cases, the mechanisms by which they interact with their molecular targets is still unclear. With respect to eNOS, the overall published data indicate that H<sub>2</sub>O<sub>2</sub> can both stimulate (Drummond *et al.* 2000) and inhibit (Wedgwood & Black 2005, Kumar *et al.* 2008) eNOS expression and stimulate (Thomas *et al.* 2001, Aschner *et al.* 2007, Tian *et al.* 2010) and inhibit (Miyamoto *et al.* 1996) eNOS activity. Of particular importance for eNOS is the kinase, pp60<sup>Src</sup>, that is the prototype non-receptor tyrosine kinase and is an important signaling molecule with many functions. pp60<sup>Src</sup> can phosphorylate phospholipase C (Marrero *et al.* 1995), complex with the epidermal growth factor receptor (Eguchi *et al.* 1998), the focal adhesion protein, paxillin (Ishida *et al.* 1999), the janus kinase-2 (Sayeski *et al.* 1999), and mediate the activation of MAP kinases (Abe *et al.* 1997). Furthermore, pp60<sup>Src</sup> is activated by H<sub>2</sub>O<sub>2</sub> leading to an increase in phosphorylation at Y418 (the autophosphorylation site) and Y215 (the SH<sub>2</sub>-domain) that are inhibited by antioxidants (Abe *et al.* 1997, Griendling *et al.* 2000, Ushio-Fukai *et al.* 2001). The activation of pp60<sup>Src</sup> by H<sub>2</sub>O<sub>2</sub> stimulates eNOS activity via PI3 kinase and AKT1 (Thomas *et al.* 2001). However, the effect of H<sub>2</sub>O<sub>2</sub> on eNOS activity appears to be both concentration dependent and time dependent with lower levels stimulating activity (Aschner *et al.* 2007, Tian *et al.* 2010) and higher levels inhibiting the enzyme (Miyamoto *et al.* 1996). The effect of H<sub>2</sub>O<sub>2</sub> on eNOS activity may also be developmentally regulated (Wedgwood & Black 2005). When taken together, previously published studies from a number of other groups suggest that there may also be differences in response to H<sub>2</sub>O<sub>2</sub> depending on the vascular bed. Related to these responses are the endogenous cellular defense mechanisms including both small molecular weight antioxidants (vitamins C and E, reduced GSH, etc.) and antioxidant enzymes. These include CuZn- and Mn-SOD,

glutathione peroxidase, and especially, catalase. Recent data has shown that these antioxidant defense mechanisms, especially catalase, can be compromised under conditions of endothelial dysfunction (Sharma *et al.* 2007). Thus, the effect of H<sub>2</sub>O<sub>2</sub> on eNOS can be separated into either a physiologic response (if catalase levels are maintained) or a pathologic response (when catalase levels are compromised). Thus, under the physiologic conditions of acute increases in oxidative stress, eNOS will be activated through S1177 phosphorylation (Fig. 3, right), while under sustained oxidative stress, eNOS dimerization will be compromised and NO signaling will be attenuated (Fig. 3, left).

### The flexible arm of eNOS: a new regulatory region

The molecular mechanism by which ZnS<sub>4</sub> cluster oxidation results in eNOS inactivation and monomerization still remains under investigation (Chen *et al.* 2010). The ZnS<sub>4</sub> cluster is formed by four covalent bonds at the dimeric interface of eNOS. The ZnS<sub>4</sub> cluster is ~20 Å away from the catalytic center (Fig. 1A); this makes it unlikely that posttranslational modification of the cysteine residues in ZnS<sub>4</sub> could directly affect eNOS activity. Despite the distance between the ZnS<sub>4</sub> cluster and the eNOS catalytic center, enzymatic activity is inhibited by ZnS<sub>4</sub> disruption suggesting that this region provides significant conformational changes within eNOS (Zou *et al.* 2002, Ravi *et al.* 2004, Tummala *et al.* 2008). Thus, other regions of the protein are implicated in regulating enzyme activity. The crystal structure of eNOS (PDB ID 3NOS; Fischmann *et al.* 1999) lacks the region located between amino acids 105–125 suggesting a general flexibility within this region. We have termed this sequence the ‘flexible arm’. Utilizing available structures of bovine eNOS (pdb ID – 1FOO), we reconstructed the 105–125 amino acid region of human eNOS (Fig. 1A). Then, to evaluate potential conformational changes in the flexible arm after ZnS<sub>4</sub> disruption, we utilized a molecular dynamic (MD) approach. ZnS<sub>4</sub> disruption was carried out by removing the Zn atom from the control structure. MD simulations were performed for both structures (with and without the Zn atom) using the same initial parameters. We identified structural changes in the flexible arm region when the normal and ZnS<sub>4</sub>-disrupted eNOS structures were superimposed (Fig. 1B). The MD simulations predicted that disruption of the ZnS<sub>4</sub> cluster results in the movement of the flexible arm (blue coil Fig. 1B) to block the access of substrate to the catalytic site (red arrow).

To test whether ZnS<sub>4</sub> disruption affects access to the heme binding site, we purified three ZnS<sub>4</sub> cluster mutants (C94A, C99A, and C94/99A) and identified a loss of a dimeric status in each of the mutant proteins as determined by analytic gel filtration (Fig. 4A). We then subjected the proteins to spectral analysis. We found that each protein contained a Soret band in the visible region characteristic of heme containing enzymes (Fig. 4B). The wild-type eNOS, in the presence of L-arginine and BH<sub>4</sub> in the active site, has a Soret band maximum at 396 nm corresponding to high-spin iron (Berka *et al.* 2008). This absorption maximum is affected by a charge transfer from the sulfur of the cysteine at position 184 to the iron of the heme (see insert Fig. 4B). It has been previously described that NOS enzymes lacking L-arginine and BH<sub>4</sub> exhibit a shift in the Soret band to an absorption maximum at 412 nm, indicative of a low-spin iron state (Berka *et al.* 2008). Interestingly, we observed the same Soret shift to 412 nm for all the ZnS<sub>4</sub> mutants (Fig. 4B). This may be explained by hindrance of heme access for L-arginine and BH<sub>4</sub> in ZnS<sub>4</sub> cluster mutants. To test the accessibility of the heme in these mutants, we conducted cyanide and imidazole binding experiments. Cyanide is a very small molecule that can easily access the heme cavity, and by binding to the heme, it produces a strong absorption in the u.v.–VIS region of the enzyme’s spectrum (Yadav *et al.* 2003). We measured the iron–cyanide complex formation for the wild-type eNOS, as well as the C94A, C99A, and C94/99A eNOS mutants using increasing concentrations of cyanide. Our data indicate that cyanide binding to wild-type eNOS resulted in a binding-type equilibrium curve with saturation point around 10 mM



KCN (Fig. 5A). Surprisingly, none of the ZnS<sub>4</sub> cluster mutants exhibited detectable cyanide binding (Fig. 5B–D). In addition, we tested imidazole binding to the heme in ZnS<sub>4</sub> mutants. Imidazole competes with L-arginine for binding to the heme. Our data indicate that although imidazole was able to dose dependently bind to the heme site in wild-type eNOS (Fig. 6A), it was unable to bind to any of the ZnS<sub>4</sub> cluster mutants (Fig. 6B–D). It is possible that the disruption of the ZnS<sub>4</sub> cluster alters either co-factor binding or the structure of the heme such that cyanide or imidazole cannot bind efficiently. To test this possibility, we utilized two other mutant proteins. The W445A eNOS mutant has an altered BH<sub>4</sub> binding ability (Joshi & Bauer 2008) while the C184A mutant removes the cysteine thiolate that acts as the proximal heme ligand (Chen *et al.* 1994). Our data indicate that both C184A (Fig. 7A and C) and W445A (Fig. 7B and D) have efficient cyanide and imidazole binding. These data provide clear evidence that the heme is accessible to both cyanide and imidazole in these mutant proteins. Thus, alterations in either the heme environment or the alterations in BH<sub>4</sub> binding are unlikely to explain the lack of cyanide or imidazole binding in the ZnS<sub>4</sub> cluster mutant eNOS proteins. Although we did not investigate the access of other small molecules such as oxygen, NO, or H<sub>2</sub>O<sub>2</sub>, it is likely that the access to the heme would also be attenuated. This suggests that the disruption of ZnS<sub>4</sub> cluster is more important in regulating accessibility to the heme versus altering BH<sub>4</sub> binding affinity or an altered heme state.

We next determined the effect of the ZnS<sub>4</sub> cluster mutants on NO and superoxide generation compared to the wild-type protein. We found almost a total loss of NO production in C94A, C99A, and C94/99A mutants (Fig. 8A) and a significant reduction in superoxide production (Fig. 8B), suggesting that a functional ZnS<sub>4</sub> cluster is required for both NO synthesis and eNOS uncoupling. These data are consistent with previous studies (Zou *et al.* 2002, Ravi *et al.* 2004). Further MD simulations predicted that the flexible arm movement is driven by formation of salt bridges between the flexible arm and the substrate channel wall. These simulations further predict that interactions between three positively charged amino acids situated in the flexible arm (Arg107, Lys108, and Arg112; Fig. 9) and negatively charged amino acids from the adjacent surface (Asp264, Glu272, Asp478, and Glu75) are the driving force in the closure of the substrate channel (Fig. 9). To test this, we generated a flexible arm mutant in which all the possible sites of salt bridges predicted from our MD simulation were mutated in combination with a disrupted ZnS<sub>4</sub> mutation. Thus, to the C94A/C99A mutant, we added R107A, K108A, and R112A mutations. We then determined how the flexible arm mutations affected dimerization using analytical gel filtration. Our data indicate that this mutant exhibits partial eNOS dimer restoration (Fig. 10A), suggesting that interactions of the flexible arm with the wall of the substrate channel can overcome the need for the ZnS<sub>4</sub> cluster in forming a dimer. Our data also indicate that the Soret band of the flexible arm mutant has a maximum at 395 nm, indicative of BH<sub>4</sub> and L-arginine binding to the heme (Fig. 10B). In addition, the mutation of positive amino acids on the flexible arm prevents the substrate channel from being blocked as both cyanide binding and imidazole were able to dose dependently bind to the protein (Fig. 10C and D). Indeed the equilibrium curve obtained for KCN binding (Fig. 10C) was similar to that of the wild-type protein (Fig. 5A). Furthermore, although the C94A/C99A mutant lacks the ability to generate NO, mutation of the flexible arm restores NO production to levels found in the wild-type enzyme (Fig. 10E). However, superoxide generation by the flexible arm mutant was increased by twofold compared with the WT eNOS (Fig. 10F), indicating that a significant amount of eNOS uncoupling is occurring. This also resulted in the flexible arm mutant generating significant peroxynitrite (Fig. 10G) that resulted in enhanced self-nitration of eNOS (Fig. 10H). The increase in superoxide generation in the flexible arm mutant may be due either to increased BH<sub>4</sub> dissociation from the heme pocket or perhaps an increase in oxygen intake due to the inability of the mutant proteins to close the substrate channel. However, the generation of NO and superoxide in close proximity results in the production of ONOO<sup>-</sup>, which results in increased self-nitration of the flexible arm mutant protein. Thus, our data

suggest that the main role of the flexible arm is to regulate the heme access of molecular oxygen in order to prevent the enhanced superoxide generation and subsequent ONOO<sup>-</sup> formation that ZnS<sub>4</sub> cluster disruption would otherwise produce. Thus, under L-arginine and BH<sub>4</sub>-saturated conditions, the flexible arm is stabilized in the open position allowing access to the heme. Decreases in L-arginine or BH<sub>4</sub> levels are known to increase superoxide generation and contribute to oxidation of the ZnS<sub>4</sub> cluster. This leads to the release of the flexible arm and closure of the heme cavity. This mechanism would limit superoxide generation by eNOS. Thus, the redox-sensitive nature of the ZnS<sub>4</sub> cluster allows conformational changes within eNOS that preserves enzyme integrity under conditions of increased oxidative stress. As the oxidation of cysteines in the ZnS<sub>4</sub> is reversible, the ZnS<sub>4</sub> can be rapidly regenerated when the redox state of the cell returns to a reduced state. When the ZnS<sub>4</sub> cluster reforms, the flexible arm region of eNOS is restored to its normal position below the substrate access cavity, allowing L-arginine and BH<sub>4</sub> to bind heme and restoring the capacity to generate NO. This process allows eNOS to rapidly cycle between activated and inhibited conformations in response to the cellular redox environment and represents a new mechanism by which eNOS activity can be regulated.

## Conclusions

The mechanisms by which eNOS is posttranslationally regulated have been under active investigation for nearly two decades. However, with our discovery of the flexible arm as a regulatory control element in eNOS, it is clear that new control mechanisms are still to be identified and even previously identified regulatory mechanisms are still far from being fully elucidated. Thus, there is still much to be learned regarding how eNOS is regulated under both physiologic and pathologic conditions.

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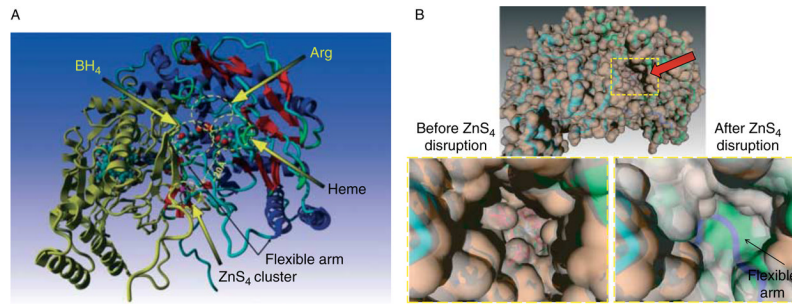
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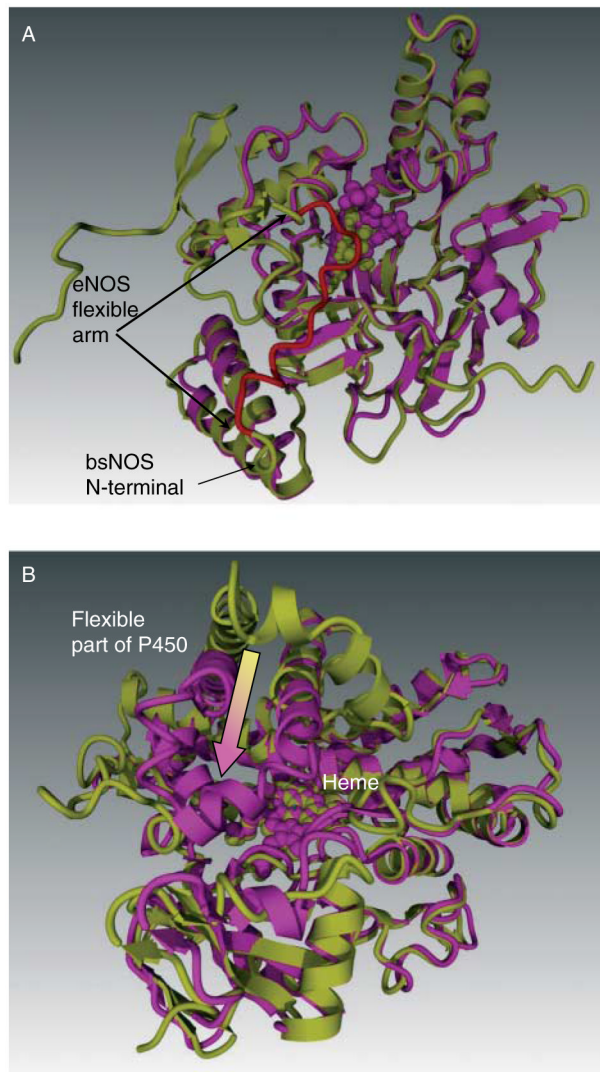


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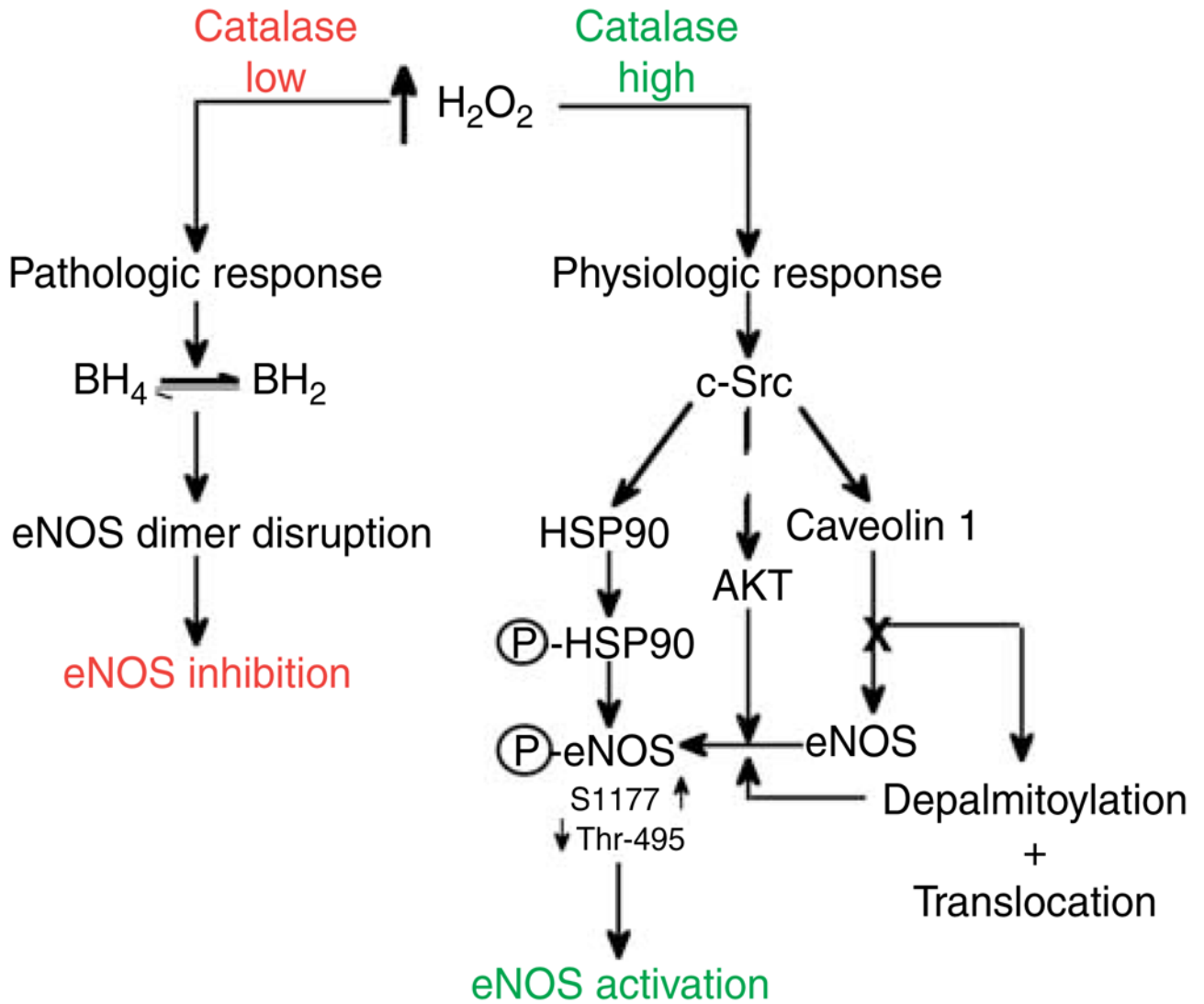
**Figure 1.**

Homology model mediated reconstruction of the flexible arm region of human eNOS. Cysteines 94 and 99 from adjacent subunits form a zinc-tetrathiolate cluster (ZnS<sub>4</sub>) that stabilizes the eNOS dimer. The ZnS<sub>4</sub> cluster appears to maintain the N-terminal region in a rigid conformation. It is also apparent that the catalytic center of eNOS is separated from the ZnS<sub>4</sub> cluster and no direct contacts are likely between them (A). Utilizing the Yasara modeling software, the ‘flexible arm’ region of human eNOS (corresponding to amino acids 105–125) was reconstructed. The ‘flexible arm’ connects the ZnS<sub>4</sub> cluster with the rest of the molecule (A). Also shown are the relative locations of the active site cavity, the ZnS<sub>4</sub> cluster, and the BH<sub>4</sub> binding site to the ‘flexible arm’ region (A). The ‘flexible arm’ appears to be seated under the cavity in the dimeric conformation with an intact ZnS<sub>4</sub> cluster. Molecular dynamic simulations predict that disruption of the ZnS<sub>4</sub> cluster will result in closure of the entrance to the catalytic cavity (arrow) of eNOS (B). Zoomed region of the structures with molecular surface are shown representing human eNOS with an intact (left) and disrupted ZnS<sub>4</sub> cluster (right) after molecular dynamic simulation (B). Flexible arm appeared in the substrate channel (blue) and blocked access to the heme after Zn removal.



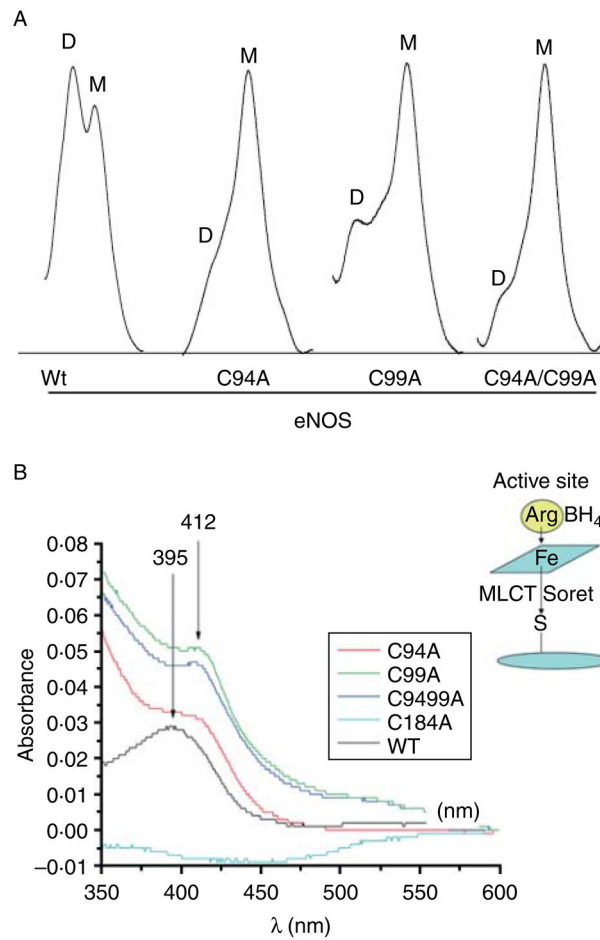
**Figure 2.**

Flexible arm feature of eNOS compared to bacterial NOS (bsNOS) and cytochrome P450. Panel A demonstrates the structural similarity of bsNOS (magenta) and eNOS (yellow). The N-terminal end of bsNOS begins immediately after the flexible arm region (red) of eNOS. Thus, bsNOS is deficient in both the redox regulation. Panel B demonstrates the superposition of open (yellow) and closed (magenta) conformations of P450. This flexible region of P450 controls substrate access to the heme and thus serves the same function as the eNOS flexible arm. However, as the P450 lacks the  $ZnS_4$  cluster, it is not susceptible to redox regulation.



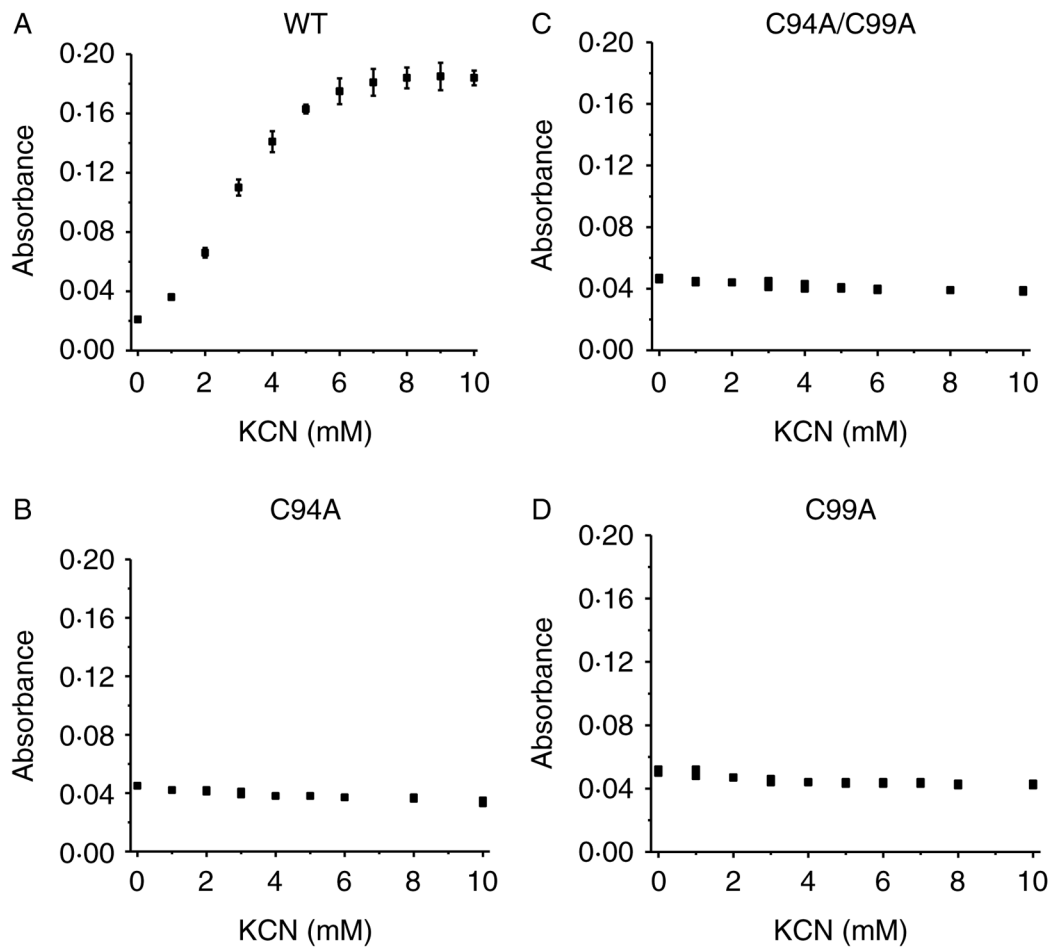
**Figure 3.**

The effect on NO signaling in endothelial cells challenged by  $H_2O_2$ . The response of EC with regard to NO generation in response to oxidative stress can be divided into two pathways: a physiologic response (right) and a pathologic response (left). The physiologic/pathologic decision is then predicated on the level of  $H_2O_2$  to which the cell is exposed, the duration of the stimulus, and the ability of the cell to metabolize it.



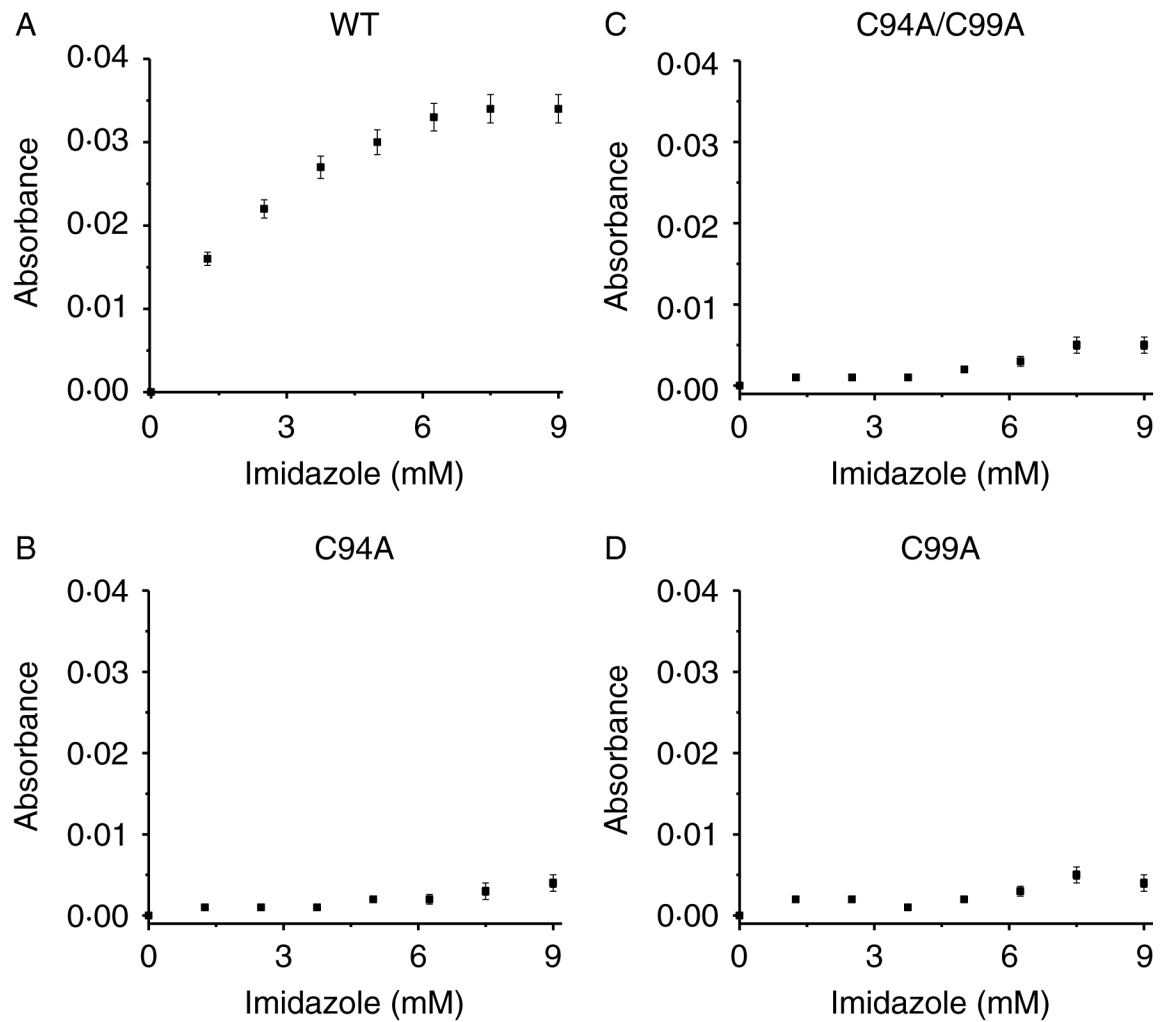
**Figure 4.**

Human eNOS ZnS<sub>4</sub> cluster mutants have reduced dimer levels. The dimer levels of human eNOS ZnS<sub>4</sub> cluster mutants purified as described (Ravi *et al.* 2004) was assessed by analytical gel filtration (A), again as described (Ravi *et al.* 2004). Analysis indicates that the dimer (D) levels are decreased and monomer (M) levels are increased in ZnS<sub>4</sub> mutants (A). Images are representative of  $n=3$ . The absorbance spectra of wild-type and ZnS<sub>4</sub> mutants were also analyzed (B). The Soret absorption maximum in wild-type eNOS is found at 395 nm and corresponds to the heme bound to L-arginine and BH<sub>4</sub> (B). A red shift in the Soret band maximum to 412 nm is observed for the ZnS<sub>4</sub> mutants corresponding to L-arginine and BH<sub>4</sub> dissociation (B). The data presented are the mean from three independent experiments. Shown in the insert is a scheme depicting the metal to ligand charge transfer (MLCT) and 'Soret band' interference (B).



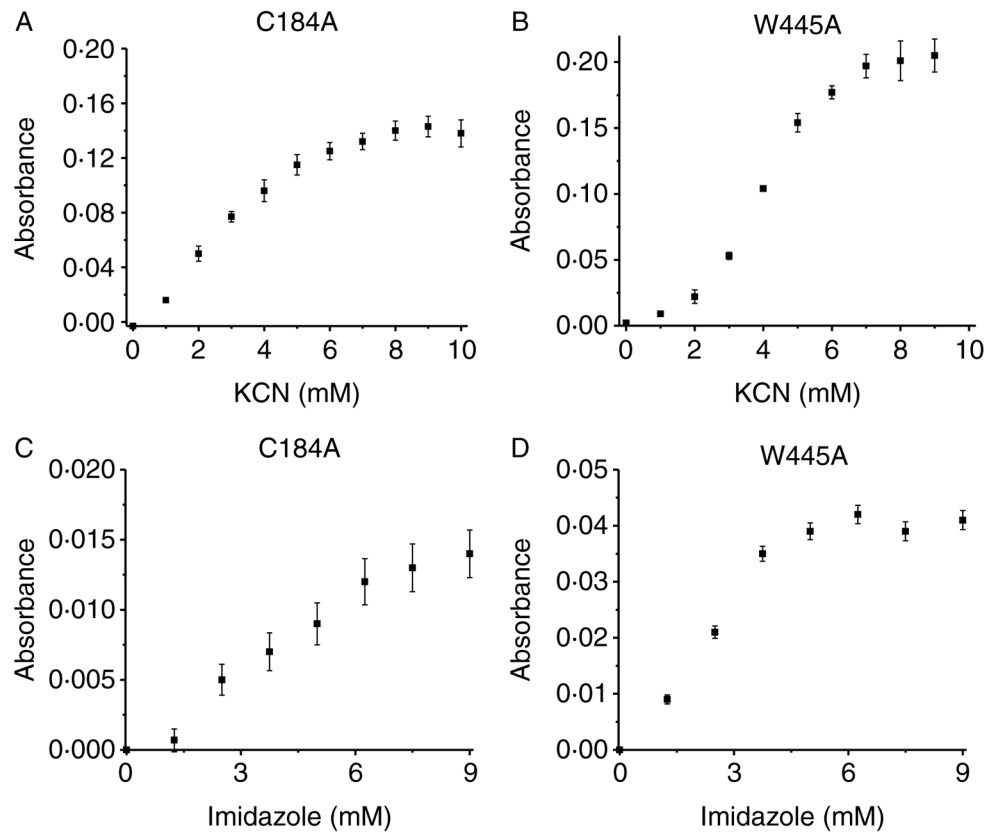
**Figure 5.**

Human eNOS ZnS<sub>4</sub> cluster mutants have reduced heme accessibility for cyanide. U.V.–visible spectra of the cyanide–iron complex were measured on a Shimadzu spectrophotometer in the region from 350 to 600 nm. Recombinant wild-type (A) and the ZnS<sub>4</sub> cluster mutants of eNOS (B–D) were dissolved in PBS to final concentration 0.1 mg/ml. The initial spectrum was obtained before addition of KCN and then the spectrum was recorded after each addition of 1  $\mu$ l KCN solution (125 mM stock). The addition of 1  $\mu$ l KCN stock solution was repeated until the reaction reached saturation, as determined by no further changes in absorption at 412 nm. There is a dose-dependent increase in KCN binding to the wild-type protein (A) that does not occur in any of the ZnS<sub>4</sub> cluster mutants (B–D). The data presented are the mean $\pm$ S.D. from three independent experiments.



**Figure 6.**

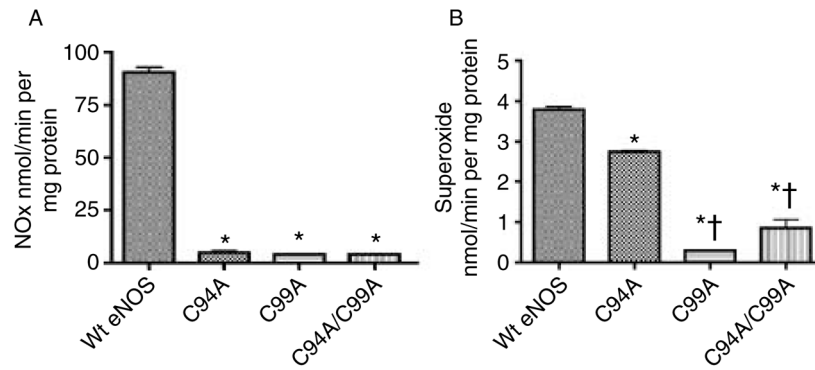
Human eNOS ZnS<sub>4</sub> cluster mutants have reduced heme accessibility for imidazole. U.V.-visible spectra of the imidazole-iron complex were measured on a Shimadzu spectrophotometer in the region from 350 to 600 nm. Recombinant wild-type (A) and the ZnS<sub>4</sub> cluster mutants of eNOS (B–D) were dissolved in PBS to final concentration 0.1 mg/ml. The initial spectrum was obtained before addition of imidazole and then the spectrum was recorded after each addition of 1  $\mu$ l imidazole solution (100 mM stock). The addition of 1  $\mu$ l imidazole stock solution was repeated until the reaction reached saturation, as determined by no further changes in absorption at 430 nm. There is a dose-dependent increase in imidazole binding to the wild-type protein (A) that does not occur in any of the ZnS<sub>4</sub> cluster mutants (B–D). The data presented are the mean  $\pm$  S.D. from three independent experiments.



**Figure 7.**

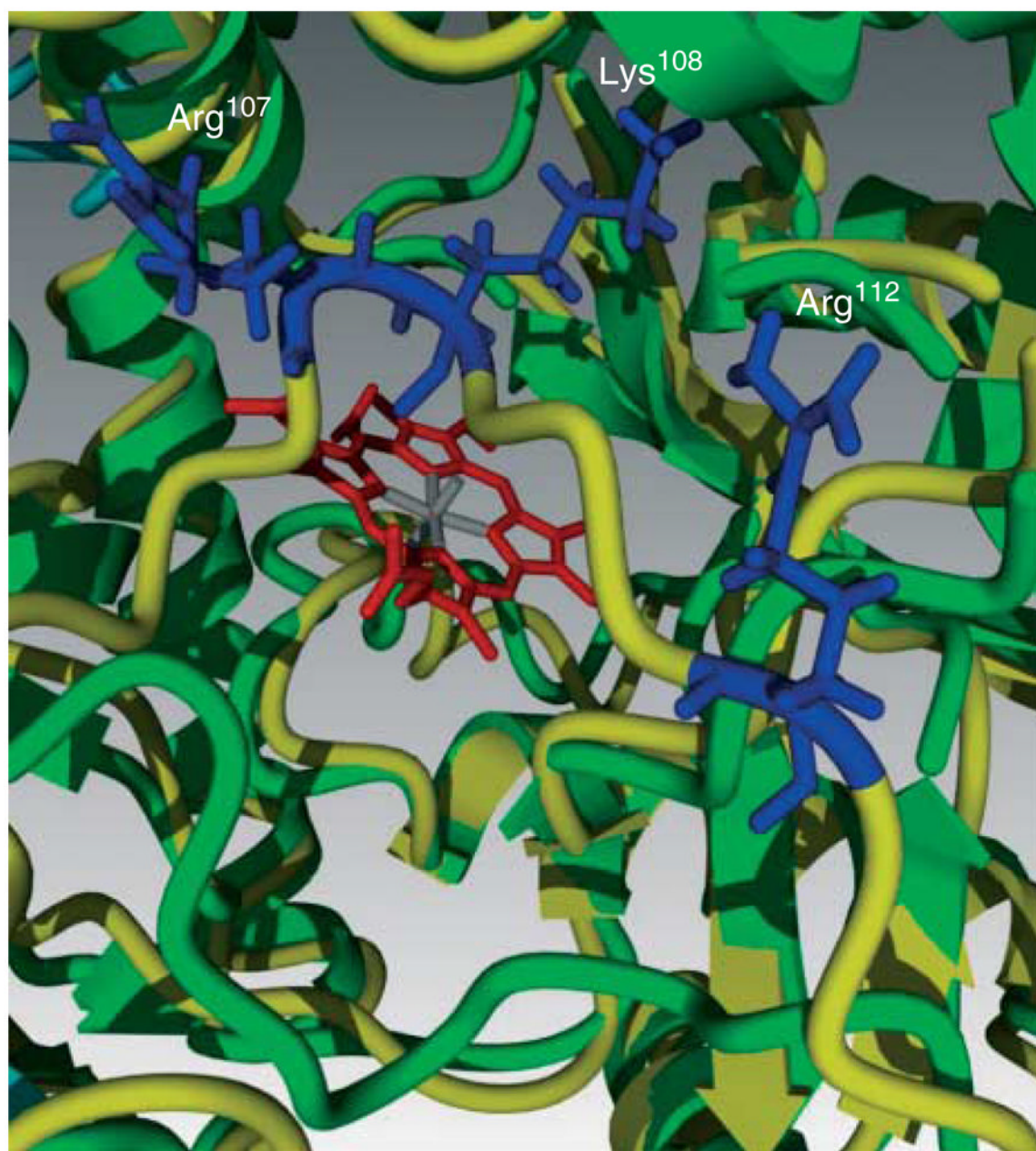
Endothelial NOS mutants with altered heme and BH<sub>4</sub> binding do not exhibit altered heme accessibility. Recombinant mutants of eNOS with altered heme (C184A) and BH<sub>4</sub> binding (W445A) were exposed to increasing concentrations of potassium cyanide (KCN, 0–12 mM) (A and B) or imidazole (C and D) as described in Fig. 6 and the changes in absorbance between 350 and 600 nm determined. There is a dose-dependent increase in both KCN and imidazole binding in the C184A- and W445A-eNOS mutant proteins. The data presented are the mean±S.D. from three independent experiments.



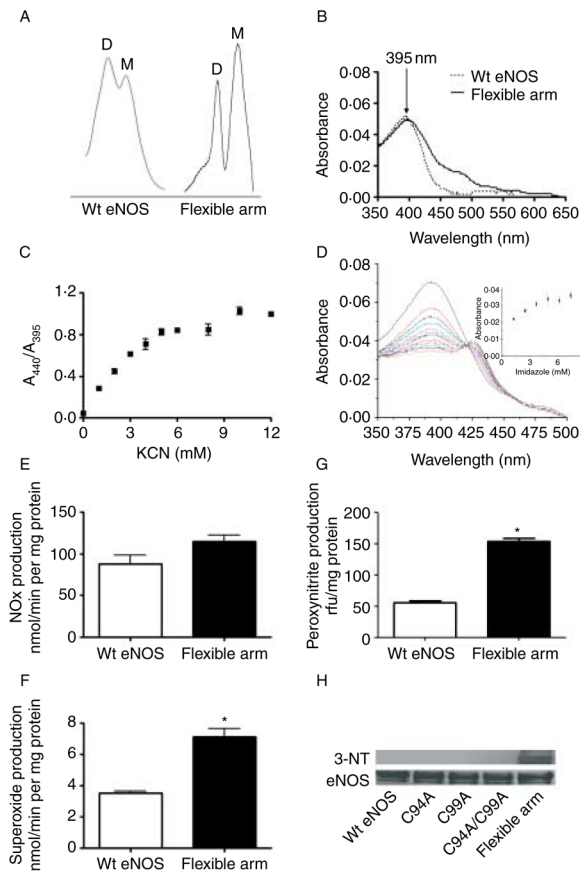


**Figure 8.**

NO and superoxide production by wild-type and the ZnS<sub>4</sub> cluster mutants of eNOS. Recombinant wild-type eNOS and the ZnS<sub>4</sub> cluster mutants (C94A, C99A, and C9499A) were incubated with L-arginine and the appropriate co-factors for 30 min at 37 °C and NO (A) and superoxide levels (B) determined as described (Grobe *et al.* 2006). There is a significant reduction in both NO (A) and superoxide (B) generation in the ZnS<sub>4</sub> cluster mutants. Data are presented as mean±S.E.M.; *n*=3. \**P*<0.05 versus wild-type eNOS. †*P*<0.05 versus C94A mutant.



**Figure 9.** Molecular dynamic simulations identify the amino acids in the flexible arm of human eNOS responsible for closure of the heme cavity. Superposition of intact eNOS (green) and with disrupted ZnS<sub>4</sub> (yellow) represents the flexible arm movement. Molecular dynamic simulations predict conformational changes in the flexible arm region that are driven by salt bridge formation between positively charged amino acids (blue) of the flexible arm (Arg107, Lys108, and Arg112) and negatively charged amino acids on the wall of the substrate channel.



**Figure 10.**

The human eNOS flexible arm mutant undergoes self-nitration. Purified wild-type recombinant eNOS was compared to the flexible arm mutant contained on a disrupted ZnS<sub>4</sub> cluster background (C94A/C99A/R107A/K108A/R112A). The flexible arm mutant has dimer (D) and monomer (M) levels, as determined by analytic gel filtration, that are similar to those seen with wild-type eNOS (wt eNOS, A). The absorbance spectrum of the flexible arm mutant contains a Soret band at 395 nm that is comparable with wild-type eNOS (B). The flexible arm mutation also allows both cyanide (C) and imidazole (D) to access the heme. The flexible arm mutant produces NO at levels comparable to the wild-type protein (E). However, the flexible arm mutant significantly generates greater levels of both superoxide (F) and ONOO<sup>-</sup> (G) compared with the wild-type protein. Western blot analysis using an antibody specific for 3-NT residues indicates that the flexible arm mutant undergoes increased self-nitration compared with either wild-type eNOS or the ZnS<sub>4</sub> cluster mutants (C94A, C99A, and C94A/99A, H). A representative image is shown. Data are presented as mean±S.E.M.; *n*=3. \**P*<0.05 versus wild-type eNOS.

**Table 1**

Alignment of conserved flexible region in endothelial NOS and mammalian P450s. Positively charged amino acids are indicated in bold

102	SLVFP <b>R</b> KLQ <b>G</b> R <b>P</b> -SPGPPAPEQLLS	125	P29474	NOS3_HUMAN
	* . . . : : * . . : * : .			
89	SWTHE <b>K</b> NW <b>K</b> KAHNILLPSFSQQAMK	113	P14779	CPXB_BACME
113	IFANGER <b>R</b> W <b>R</b> AL <b>R</b> R <b>F</b> SLATMRDFGMGK	138	P00178	CP2B4_RABIT
123	TFSPD--WA <b>A</b> <b>R</b> <b>R</b> <b>R</b> --LAQDSLKSFSI	149	P00187	CP1A2_RABIT
122	SS-NG <b>K</b> R <b>W</b> <b>K</b> E <b>I</b> R <b>R</b> FSLTTLRNFGMGK	147	P10632	CP2C8_HUMAN
124	IFNNGPTW <b>K</b> D <b>I</b> R <b>R</b> FSLTTLRNYGMGK	150	P05181	CP2E1_HUMAN