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REVIEW ARTICLE

Endothelial NOS: perspective and recent developments

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Endothelial NOS (eNOS), and its product NO, are vital components of the control of vasomotor function and cardiovascular homeostasis. In the present review, we will take a deep dive into eNOS enzymology, function and mechanisms regulating endothelial NO. The mechanisms regulating eNOS and NO synthesis discussed here include alterations to transcriptional, post-translational modifications and protein–protein regulations. Also, we will discuss the phenotypes associated with various eNOS mutants and the consequences of a disrupted eNOS/NO cascade, highlighting the importance of eNOS function and vascular homeostasis.

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Abbreviations

BH4, tetrahydrobiopterin; CaM, calmodulin; Cav-1, caveolin-1; CSD, Cav-1 scaffold domain; EC, endothelial cells; eNOS/NOS3, endothelial NOS; Hbα, haemoglobin α; LEENE, lncRNA that enhances eNOS expression; lncRNAs, long non-coding RNAs; ox-LDL, oxidized LDL; STEEL, spliced-transcript endothelial-enriched lncRNA

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Introduction

The discovery of endothelial-derived relaxing factor, identified as **NO**, revolutionized our understanding of local control of vasomotor function and cardiovascular homeostasis. The identification of **endothelial NOS (eNOS, NOS3)** as the enzyme responsible for generating endothelial-derived NO has led to the exploration of how this enzyme is regulated in health and disease. The central focus of this review is to highlight recent advances in the field and perspectives for the future.

Overview of eNOS enzymology and function

Akin to all NOS isoforms, eNOS is a homodimeric, haem-containing oxidoreductase that couples the metabolism of **L-arginine** in the oxygenase domain with **NADPH**-dependent flux of electrons from the reductase domain. Upon changes in local calcium, calcium-activated **calmodulin (CaM)** facilitates interdomain electron transfer and accelerates NO synthesis. The rates of electron transfer and haem iron reduction differ amongst NOS isoforms and requires the critical co-factor **tetrahydrobiopterin (BH4)** for optimal NO synthesis. Unlike the other NOS isoforms, eNOS is predominately membrane bound due to amino-terminal fatty acylation by myristic and palmitic acid, and dual acylation brings eNOS into the proximity of biological membranes such as the cytoplasmic face of the Golgi or plasmalemmal caveolae. The proper subcellular localization of eNOS ensures optimal regulation by mechanical forces (shear stress or pressure gradients), calcium ions and kinases. Therefore, any agonist that mobilizes intracellular calcium (**VEGF**, **bradykinin**, **histamine**, etc.) or alterations in intracellular signalling pathways leading to enhanced CaM binding or reduced CaM dissociation has the potential to promote eNOS activity and NO release. Under conditions of BH4 depletion or L-arginine deficiency, eNOS can become uncoupled and generate oxygen-derived free radicals (Forstermann and Sessa, 2012).

Activation of eNOS promotes endothelium-dependent vasodilation and regulates systemic blood pressure. Accordingly, mice lacking eNOS also lack endothelium-dependent relaxation of large blood vessels, have markedly elevated blood pressure, impaired vascular remodelling and are more susceptible to ischaemic injury, myocardial infarction and atherosclerosis, consistent with the known cardioprotective actions of the endothelial-derived NO. Although the endothelium can produce other gasotransmitters such as **carbon monoxide** and **hydrogen sulfide**, vasodilatory eicosanoids and other vasoactive lipids, these substances do not adequately compensate for the loss of eNOS in mice. Interestingly, recent data suggest that eNOS is required for the actions of hydrogen sulfide (King *et al.*, 2014) and that there are multiple levels of crosstalk between hydrogen sulfide and the NO-cGMP pathway (Bucci *et al.*, 2010; Bucci *et al.*, 2012). Despite the importance of eNOS in whole body cardiovascular homeostasis, the role of eNOS in controlling microvascular flow is less prominent. Mechanistically, eNOS-derived NO can activate soluble guanylate cyclase, thereby increasing the formation of the second messenger, **cGMP** and activation of **protein kinase G**. Protein kinase G phosphorylates several downstream substrates that are responsible for many of the biological actions of NO. Additionally, eNOS can provide NO equivalents used in additional chemical reactions throughout the body. The actions and targets of eNOS-derived NO will be reviewed in other articles in this series, and the remainder of this review will focus on regulation of eNOS gene expression and post-translational control of eNOS activity.

Transcriptional and post-transcriptional regulation of eNOS

The human eNOS gene is located on Chromosome 7 and contains a promoter region rich in several transcription factor binding sites for a variety of transcription factors including KLF2, Sp1, Sp3, Ets1,Ets2, Smad2, GATA1, GATA2, GATA4, AP-1, cJun/Fos, NFkB, CHOP10 and p53. Although eNOS was initially considered to be a constitutively expressed gene [relative to inducible NOS (iNOS) or NOS2], there are several circumstances where it can be induced several-fold. Most importantly, eNOS gene expression is up-regulated by fluid shear stress (Nishida *et al.*, 1992) and cyclic stretch (Awolesi *et al.*, 1995) in cultured endothelial cells (EC), and this has been observed also in exercised animals (Sessa *et al.*, 1994; Fukai *et al.*, 2000). Short-term changes in shear stress rapidly activate eNOS (see below section on phosphorylation), and this is critical for flow-mediated regulation of vascular tone while sustained shear promotes eNOS gene expression and chronic remodelling of blood vessels. Some additional stimuli that induce eNOS include VEGF-A (Papapetropoulos *et al.*, 1997; Bouloumie *et al.*, 1999), **TGFβ** (Saura *et al.*, 2002), lysophosphotidycholine (Zembowicz *et al.*, 1995), statins (Laufs *et al.*, 1998), **cyclosporine A** (Navarro-Antolin *et al.*, 2000), **H_2O_2** (Searles, 2006), and **oestrogen** (Tan *et al.*, 1999). Conversely, pro-inflammatory stimuli such as oxidized LDL (ox-LDL) (Liao *et al.*, 1995), **TNFα** (Nishida *et al.*, 1992; Neumann *et al.*, 2004), **LPS** (Lu *et al.*, 1996) and hypoxia (McQuillan *et al.*, 1994; Fish *et al.*, 2010) can reduce eNOS mRNA levels. The stability of eNOS mRNA has been reported to range between 24 and 48 h (Yoshizumi *et al.*, 1993; McQuillan *et al.*, 1994), and a significant decrease in eNOS mRNA stability has been observed under TNFα, ox-LDL and hypoxic conditions. Mechanistically, DNA methylation of the eNOS promoter can dramatically reduce Sp1, Sp3 and Ets1 transcription factor binding and eNOS mRNA levels (Chan *et al.*, 2004). Hypoxia promotes dramatic changes to eNOS expression through a variety of mechanisms, including changes to transcription factor dynamics, decreases in mRNA stability and most recently changes to histones associated with the eNOS promoter (Fish *et al.*, 2010). Additional work has described a dynamic 'histone code' where changes to histones at the proximal promoter of eNOS regulate eNOS mRNA levels (Fish *et al.*, 2005). This code includes the acetylation of histones H3 and H4 and the dimethylation and trimethylation of histone H3. In fact, the decrease in transcription observed in hypoxia is in part attribute to a reduction in acetylation and methylation of eNOS proximal promoter histones (Fish *et al.*, 2010).

Recently, work exploring the role of long noncoding RNAs (lncRNAs) in EC uncovered two distinct lncRNAs: spliced-transcript endothelial-enriched lncRNA (STEEL) and lncRNA that enhances eNOS expression (LEENE) that are able to influence eNOS mRNA levels. STEEL can up-regulate the important EC transcription factor KLF2 and thereby increases eNOS mRNA levels (Man *et al.,* 2018). Using a combination of transcriptome and chromatin conformation profiling, LEENE was discovered from an enhancer that has proximal association with the eNOS genomic locus (Miao *et al.,* 2018). Both STEEL and LEENE have strong ties to KLF2, an established driver of eNOS transcription (Lin *et al.,* 2005), with STEEL and LEENE not only influencing the KLF2 promoter but also relying on KLF2 at the transcriptional level. In addition to lncRNAs, a role of micro RNAs (miRNAs) on eNOS levels has been described. The loss of Dicer in EC, which blocks the synthesis of all mature miRNAs, increases eNOS mRNA and protein levels, effects rescued by miR-221/222. This effect is likelyto be indirect as there are no miR221/222 seed sequences in the eNOS 3′ untranslated region (Suarez *et al.,* 2007). mIR-92a is the best studied miRNA regulating eNOS mRNA levels via targeting the transcription factor KLF2. Indeed, antagonism of miR-92 increases KLF2 levels and eNOS activity and promotes beneficial effects on cardiac function, arteriogenesis and angiogenesis (Bonauer *et al.,* 2009).

eNOS regulation by post-translational modifications

Initially, eNOS was believed to be regulated only by the essential allosteric regulator of all NOS isoforms, calcium-activated CaM. However, detailed work on the subcellular localization od eNOS and mapping of its post-translational modifications has provided novel insights into how mechanical forces, growth factors, bioactive peptides and lipid mediators regulate NO synthesis in EC. The purification of eNOS as a membrane protein and the subsequent cloning and identification of the mechanisms of membrane targeting via acylation have led to a series of studies identifying post-translational control mechanisms such as phosphorylation and protein–protein interactions that regulate eNOS activity beyond CaM, thereby fine tuning eNOS activation. In addition to these modifications, eNOS can be S-nitrosylated (at C94 and C98) (Erwin *et al.,* 2005) reducing its activity, acetylated (K609, S765 and S771) increasing its activity (Jung *et al.,* 2010) or glutathionylated in the C-terminal reductase domain (C689 and C908) uncoupling eNOS to generated superoxide anion (Chen *et al.,* 2010).

Multisite phosphorylation of eNOS regulates activity

Work in the 1990s documented that eNOS is a phosphoprotein. In 1999, the most abundant phosphoserine site (S1176, S1177 or S1179 in murine, human or bovine eNOS, respectively) was discovered using mass spectrometry (Dimmeler *et al.,* 1999; Fulton *et al.,* 1999). The residue S1177 in human eNOS is a canonical phospho-acceptor site for the protein kinase **Akt**; however, additional AGC kinase family members (protein kinase A, protein kinase G, protein kinase C and **AMP-activated kinase; AMPK**) can phosphorylate this site *in vitro* and perhaps *in vivo*. Since the discovery of S1177, six additional phosphorylation sites have been mapped including Y81, S114, T495, S615, S633 and Y657. Investigations into these various phosphorylation sites have shown eNOS activity to be activated or inhibited depending on which particular site is phosphorylated. Moreover, there are stimulus-dependent kinetic changes in most of these sites depending on the method of stimulation and the duration of the response (Bauer *et al.,* 2003). Y81, S615, S633 and S1177 have been identified as stimulatory sites for eNOS activity while S114, T495 and Y657 are recognized as inhibitory sites (Figure 1). Src kinase is the dominant kinase phosphorylating Y81, thus increasing eNOS activity and NO production (Fulton *et al.,* 2005). The kinases PKA, AMPK and Akt have been implicated in the phosphorylation of the S615 and S633 enhancing enzyme activity (Fulton, 2016). The inhibitory site S114 is increased under conditions of shear stress and influenced by **ERK** while the Y495 site has been shown to be phosphorylated by AMPK, **ROCK** and PKC (Navarro-Antolin *et al.,* 2000). Most recently, proline-rich proline-rich tyrosine kinase 2 (**PYK2**) phosphorylates Y657 and impairs eNOS enzyme activity (Loot *et al.,* 2009).

Despite the presence of many sites for the phosphorylation of eNOS, there has been little mechanistic work to characterize how each site directly regulates enzymic activity, using purified eNOS. Early work demonstrated that eNOS contained two autoinhibition sites, near the CaM binding domain and at the extreme carboxy terminus of the protein and that putative phosphorylation sites were in the vicinity of these inhibitory sites (Salerno *et al.,* 1997). As shown for the S1179 site (bovine eNOS) within the carboxy-terminal inhibitory domain, mutation of S1179 to aspartate (eNOS S1179D) renders eNOS constitutively active and coupled suggesting that the negative charge of phosphate or aspartate dampens autoinhibition. Indeed, purified eNOS S1179D is more active than non-phosphorylated eNOS and has enhanced electron flux from the reductase to the oxygenase domain and reduced CaM dissociation at lower concentrations of calcium (McCabe *et al.,* 2000).

Although there is ample *in vitro* evidence for each of the phosphorylation sites influencing eNOS activity, the only definitive *in vivo* evidence supporting a physiological role of eNOS phosphorylation is for S1176 (murine site), because the relevant mutant mice are available. Using a gene-targeting strategy, mice were generated expressing a serine to alanine mutation (eNOS S1176A) or serine to aspartate mutation (eNOS S1176D; Figure 2). Biochemically, eNOS S1176A cannot be phosphorylated and eNOS S1176D is a 'gain of function' mutant. Interestingly, eNOS S1176A mice exhibit reduced endothelial-dependent responses, enhanced blood pressure, insulin resistance and increased weight. These effects are diminished in eNOS S1176D mice that demonstrate enhanced endothelial-dependent responses and are protected from insulin resistance and stroke (Atochin *et al.,* 2007; Kashiwagi *et al.,* 2013; Li *et al.,* 2013). Interestingly, atherosclerotic mice lacking the ApoE gene have larger lesions when bred to eNOS S1176A mice (Park *et al.,* 2016), similar to eNOS-deficient mice bred to

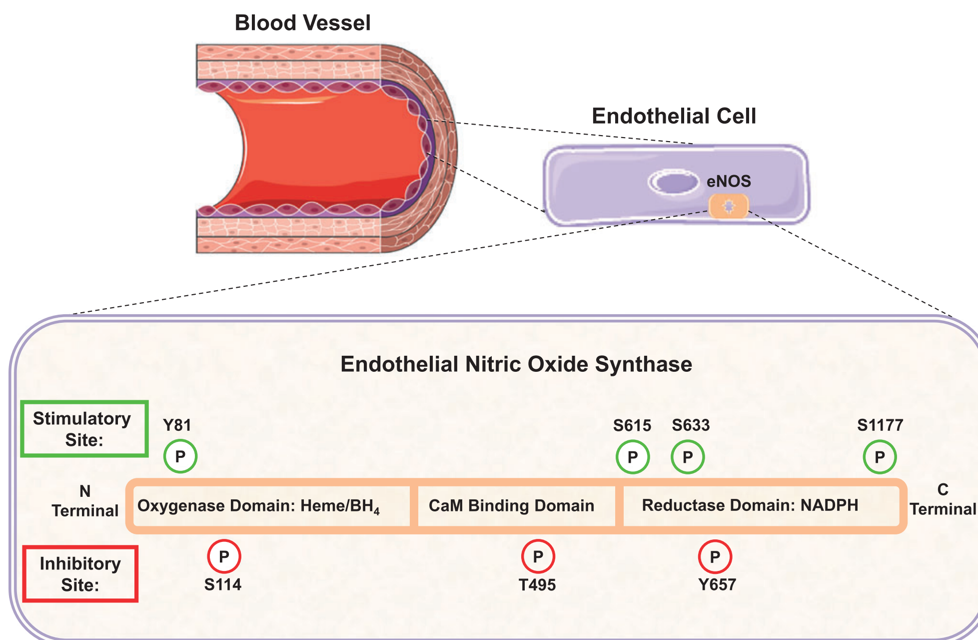

Figure 1

Diagram illustrating the various phosphorylation sites (stimulatory and inhibitory) located in human eNOS.

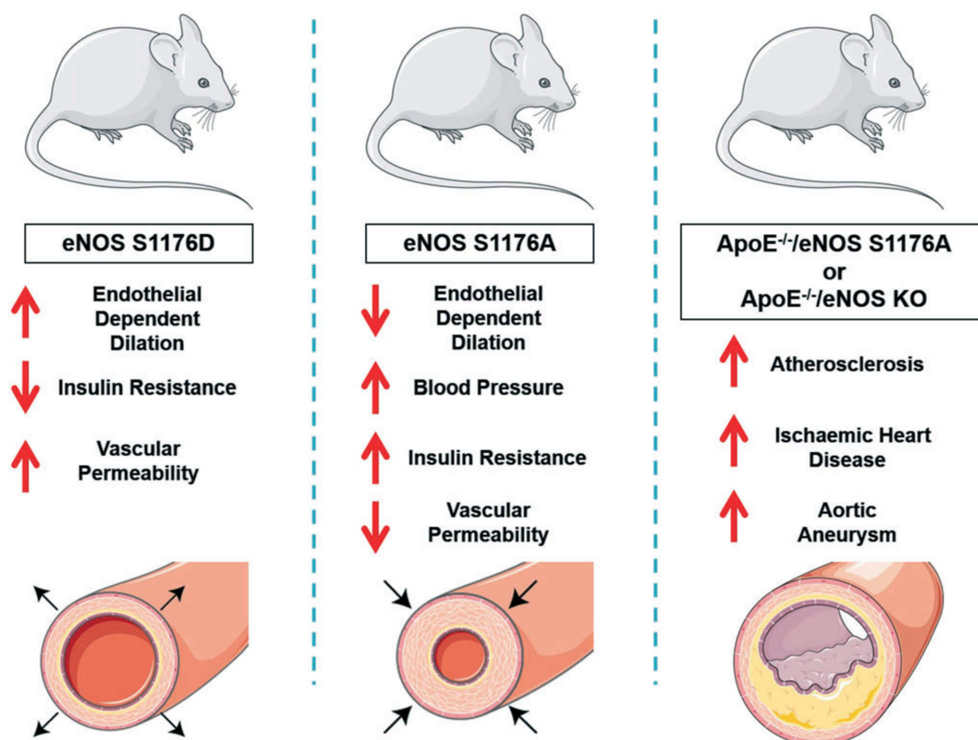

Figure 2

Diagram illustrating the phenotypes observed in eNOS mutant mice including eNOS S1176D, eNOS S1176A and the double mutant $ApoE^{-/-}$/eNOS S1176A and $ApoE^{-/-}$/eNOS KO mice.

the same atherosclerosis-prone strain (Kuhlencordt *et al.,* 2001). Phosphorylation of S1176 in eNOS is also critical for vascular permeability changes induced by VEGF, histamine and non-immune irritants, as eNOS S1176A mice show diminished permeability while eNOS S1176D mice show normal or enhanced changes in vascular leakage (Di Lorenzo *et al.,* 2013).

As mentioned before, many kinases can phosphorylate eNOS at S1176, and the importance of each kinase *in vivo* is virtually unknown. In order to test the physiological importance of Akt as an eNOS kinase, global Akt1-deficient mice were bred to eNOS mutant mice (Schleicher *et al.,* 2009). Notably, eNOS S1176D mice rescued impaired wound healing and ischaemic angiogenesis typically observed in Akt1-deficient mice; effects attenuated in Akt1-deficient/eNOS S1176A mice. Thus, this genetic epistasis experiment implies that eNOS is an important Akt1 substrate *in vivo* and that S1176 is a critical site of eNOS phosphorylation, independent of the additional sites of phosphorylation.

Flow or shear-induced eNOS activation is one of the most important physiological stimuli regulating acute changes in blood flow and long-term vascular remodelling. Increases in blood flow (and the attendant changes in shear stress) can activate the endothelial cation channel, **PIEZO1**, promoting ATP release and activation of the purinergic receptor, **$P2Y_2$**, which is coupled to the G protein, G11. Activation of this pathway stimulates Akt phosphorylation and eNOS phosphorylation on S1176 (Wang *et al.,* 2016). Consequently, mice lacking PIEZO1 in endothelium have reduced NO formation and eNOS activation and develop hypertension. Recently, endothelial-specific Akt1-deficient mice were generated, and these mice have reduced circulating NO levels, reduced eNOS S1176 phosporylation in blood vessels, increased blood pressure and impaired vessel function and ischaemic arteriogenesis (Lee *et al.,* 2018). However, these deficits were not as severe as eNOS or global Akt1-deficient mice suggesting additional levels of eNOS regulation by additional kinases. Similar genetic epistasis experiments are clearly needed for other eNOS kinases, and mutant strains lacking the other sites of eNOS phosphorylation are critical to delineate the complex role of eNOS phosphorylation *in vivo*.

Regulation of eNOS by protein–protein interactions

In addition to the dynamic, post-translational regulation of eNOS, there is extensive evidence demonstrating the importance of additional proteins that stimulate or inhibit eNOS function. The most important protein regulator of eNOS is CaM. Calcium-activated CaM regulates all NOS isoforms and is an essential allosteric regulator facilitating NADPH-dependent electron flux from the reductase domain to the oxygenase domain. In endothelial-lined vessels and in cultured EC, any agonist that triggers an influx of extracellular calcium or mobilizes intracellular calcium can promote endothelial-dependent relaxations and NO release. Early evidence suggested that fluid shear stress can induce calcium-independent NO release since neutralization of either extracellular or intracellular calcium did not affect shear-induced NO release and sustained shear did not mobilize intracellular calcium, as measured with fluorometric dyes. However, it was subsequently shown that low levels of calcium-activated CaM can bind eNOS in a manner similar to iNOS and that phosphorylation of eNOS may change the association and dissociation rates of CaM binding to eNOS (McCabe *et al.,* 2000). In addition, there is evidence that the binding of **heat shock protein 90** to eNOS regulates the sensitivity of CaM towards eNOS (Brouet *et al.,* 2001; Fontana *et al.,* 2002). As mentioned above, there are many eNOS interacting proteins and this topic has been extensively reviewed recently (Siragusa and Fleming, 2016). However, we will highlight work on caveolin-1 (Cav-1) and haemoglobin α (Hbα) as these interactions that have been best characterized *in vitro* and are physiologically relevant *in vivo*.

eNOS regulation and interaction with caveolin-1

Caveolae are flask-shaped invaginations of the plasma membrane that are highly enriched in endothelia. Indeed, caveolae are the major plasmalemmal vesicle in EC and are prominent in all endothelia lining large and small blood vessels. Cav-1 is the key protein required for the formation of caveolae since the genetic loss of Cav-1 eliminates all measurable caveolae (Drab *et al.,* 2001; Razani *et al.,* 2001). In addition to Cav-1, additional proteins called cavins were discovered which modify caveolae number and function (Chidlow and Sessa, 2010). Early insights into the eNOS–Cav-1 connection was determined by co-localization and co-fractionation of eNOS with Cav-1 (Garcia-Cardena *et al.,* 1996; Shaul *et al.,* 1996)). Moreover, experiments with purified components showed that eNOS can interact with at least two domains of Cav-1 and this interaction negatively regulated eNOS activity and NO release from cells. One juxtamembrane domain of Cav-1, termed the Cav-1 scaffolding domain (CSD), was shown to be sufficient for eNOS docking and inhibition of eNOS activity. One issue that is still unresolved *in vivo* is whether eNOS directly interacts with Cav-1 *in vivo*, although *in vitro* experiments support this model (Garcia-Cardena *et al.,* 1997; Ju *et al.,* 1997). Physiologically, the interaction of eNOS is functionally relevant since one of the main phenotypes in mice lacking Cav-1 is enhanced endothelium-dependent relaxations, cardiac and pulmonary vascular changes and cardiomyopathy (Drab *et al.,* 2001; Razani *et al.,* 2001; Murata *et al.,* 2007). Paradoxically, although acetylcholine-mediated relaxations are enhanced, flow-induced dilation and proportional vascular remodelling are attenuated (Yu *et al.,* 2006). This deficit in flow-mediated mechanosignalling is now linked to the role of caveolae as source of residual plasma membrane that dynamically respond to alterations in membrane tension (Sinha *et al.,* 2011).

Interestingly, generation of a cell permeable version of the CSD has been used as a Cav-1 mimetic in experimental models *in vivo*. Early work demonstrated that treatment of mice with cell permeable CSD reduces acute changes in

vascular permeability in response to non-immune irritants (Bucci *et al.,* 2000). Since that time, several papers have shown that the CSD can reduce tissue oedema, tumour growth, asthma, retinal inflammation angiogenesis and neuroinflammation (Kraehling and Sessa, 2017). Mechanistically, eNOS can contribute to these underlying processes, but it is likely that additional Cav-1 targets are influenced by the CSD and additional work is needed to identify the molecular targets of the CSD *in vivo*.

Haemoglobin α

The relationship between the amount of NO generated from NOS and the biological actions of authentic NO gas can be less than stoichiometric. Recent evidence showing that EC express the α subunit of Hbα suggests that the biological activity of NO can be decreased through sequestration by Hb (Straub *et al*., 2012). Hbα is localized at the interface between EC and smooth muscle cells (called myoendothelial junctions) in resistance arteries, and Hbα can directly interact with eNOS and sequester NO when Hb is in its high affinity, reduced Fe^{2+}-form. This binding results in the oxidation of Hbα to the Fe^{3+} state and the generation of nitrate and the cycling of the Fe ions back to the reduced state is via the enzyme NADH-cytochrome b_5 reductase 3. The Fe^{3+} Hbα has a much lower affinity for NO, thereby permitting the limited diffusion of NO to the underlying vascular smooth muscle. Pharmacological manipulation of this pathway using a novel peptide to disrupt the interaction of eNOS with Hbα reduces blood pressure in a model of hypertension (Straub *et al*., 2014).

Concluding remarks and future perspectives

The discovery of a unique NOS isoform regulating endothelial-dependent responses has led to an explosion of information on the molecular and cellular pathways regulating eNOS function. In experimental models and in humans, eNOS is critical for normal vascular homeostasis, vascular remodelling and adaptation to stress and exercise. In most cardiovascular diseases, impaired endothelium-dependent responses is a hallmark of disease progression, but it is not clear if correction of this abnormality in humans would delay or prevent disease. Perhaps a deeper understanding of the multifaceted regulation of eNOS will permit the identification of new therapeutic approaches for the treatment of vascular dysfunction and disease.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.,* 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.,* 2017a,b,c).

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Conflict of interest

The authors declare no conflicts of interest.

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