Mechanisms and consequences of endothelial nitric oxide synthase dysfunction in hypertension

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See editorial comment on page 1137

Reduced nitric oxide bioavailability contributes to endothelial dysfunction and hypertension. The endothelial isoform of nitric oxide synthase (eNOS) is responsible for the production of nitric oxide within the endothelium. Loss of eNOS cofactor tetrahydrobiopterin to initial increase in oxidative stress leads to uncoupling of eNOS, in which the enzyme produces superoxide anion rather than nitric oxide, further substantiating oxidative stress to induce vascular pathogenesis. The current review focuses on recent advances on the molecular mechanisms and consequences of eNOS dysfunction in hypertension, and potential novel therapeutic strategies restoring eNOS function to treat hypertension.

Keywords: endothelial dysfunction, endothelial nitric oxide synthase, endothelial nitric oxide synthase uncoupling, hypertension, nicotinamide adenine dinucleotide phosphate oxidase, nitric oxide, oxidative stress, tetrahydrobiopterin

Abbreviations: 6R-H_4B, sapropterin dihydrochloride; AAA, abdominal aortic aneurysm; ACE, angiotensin-converting enzyme; ADMA, asymmetric dimethylarginine; Ang II, angiotensin II; BAEC, bovine aortic endothelial cell; Cav-1, caveolin-1; cGMP, 3′,5′-cyclic-guanosine monophosphate; CKD, chronic kidney failure; DHFR, dihydrofolate reductase; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GTPCHI, GTP cyclohydrolase I; H_2B, dihydrobiopterin; H_4B, tetrahydrobiopterin; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; NOX, NADPH oxidase; PKG, cGMP-dependent protein kinase; SHR, spontaneously hypertensive rat; SPR, sepiapterin reductase

INTRODUCTION

There are at least 970 million people worldwide suffering from hypertension [1]. Patients with high blood pressure develop more cardiovascular complications [2]. Globally, cardiovascular disease accounts for approximately 17 million deaths a year – nearly one-third of the total mortality [3]. Among these, complications of hypertension account for 9.4 million deaths worldwide every year [3,4]. It has become clear that nitric oxide, produced by the endothelial isoform of nitric oxide synthase (eNOS) in the vascular endothelium, plays an important role in regulating blood pressure. Reduced nitric oxide bioavailability, which is considered a hallmark of endothelial dysfunction [5], plays an important role in mediating blood pressure elevation. Endothelial dysfunction also predicts atherosclerotic coronary and cerebral artery disease in hypertension. A better understanding of the molecular mechanisms regulating nitric oxide signaling under pathophysiological conditions is critically important in designing new therapeutic options. Therefore, in the present review, we will discuss the following aspects of nitric oxide signaling that are relevant to hypertension: nitric oxide and blood pressure regulation; mechanisms of eNOS dysfunction; consequences of eNOS uncoupling in hypertension; and potential novel therapies targeting uncoupled eNOS in hypertension.

NITRIC OXIDE AND BLOOD PRESSURE REGULATION

Accumulating evidence demonstrates a critical role of nitric oxide in blood pressure regulation. Released from the endothelial cells, nitric oxide increases 3′,5′-cyclic-guanosine monophosphate (cGMP) production and subsequent cGMP-dependent protein kinase (PKG) activation in the underneath vascular smooth muscle cells (VSMCs), resulting in vasodilatation [6,7]. Pathophysiological regulation of nitric oxide signaling in endothelial cells and its relevance to VSMC regulation are summarized in Fig. 1. Previous studies have confirmed an essential role of nitric oxide in vasorelaxation of large human arteries [8]. In addition, impairment in nitric oxide-mediated vasodilatation in brachial, coronary, and renal arteries was also observed in patients with essential hypertension [9–12]. Moreover, the relaxation mediated by nitric oxide was depressed in mesenteric arteries of hypertensive rats with reduced renal mass, as the result of an arterial endothelial abnormality

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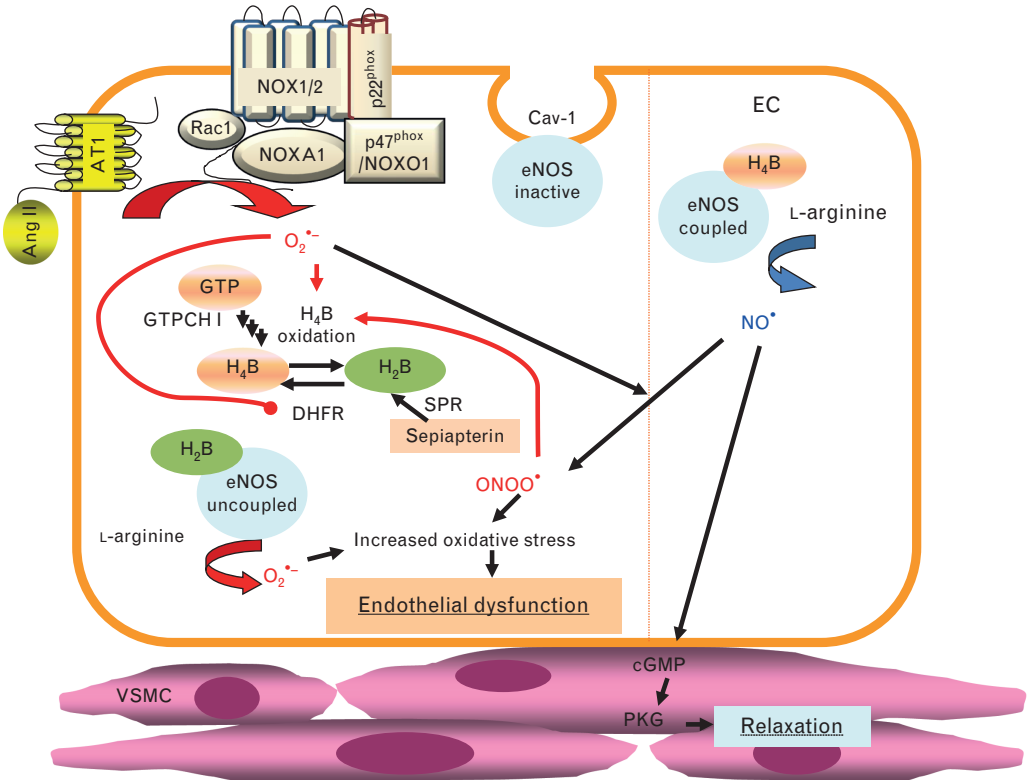

FIGURE 1 Endothelial dysfunction by endothelial nitric oxide synthase (eNOS) uncoupling. eNOS produces nitric oxide (NO) to mediate vasorelaxation and preservation of vascular function. Tetrahydrobiopterin (H_4B) is the key cofactor responsible for normal electron transfer from the reductase domain of one eNOS monomer to the oxygenase domain of the other monomer to produce NO, and in its deficiency, eNOS produces superoxide rather than NO, a process now referred to as eNOS uncoupling. H_4B can be supplied by both de-novo biosynthesis and salvage pathways. It is newly synthesized from GTP by activations of sequential enzymes of GTP cyclohydrolase I (GTPCH I), PTP synthase (PTPS), and sepiapterin reductase (SPR), or restored from its oxidized form H_2B by the salvage enzyme dihydrofolate reductase (DHFR). SPR can also catalyze conversion of H_4B precursor sepiapterin to dihydrobiopterin (H_2B), prior to its conversion to H_4B by DHFR. Pathological stimuli such as Angiotensin (Ang) II activates NADPH oxidase isoform 1 (NOX1) to produce superoxide in endothelial cells, which in turn causes peroxynitrite-dependent oxidation of H_4B and hydrogen peroxide-dependent DHFR deficiency, leading to persistent reduction in H_4B bioavailability. Superoxide production by uncoupled eNOS further sustains oxidative stress in the vasculature, resulting in endothelial dysfunction, impaired endothelium-dependent vasorelaxation, and elevated blood pressure.

[13,14]. In high-salt-treated Dahl hypertensive rats, eNOS mRNA expression was downregulated in mesenteric arterioles [15]. In deoxycorticosterone acetate (DOCA)-salt hypertensive rats, reduced eNOS phosphorylation resulted in reduced nitric oxide/cGMP signaling in mesenteric arteries [16]. Taken together, nitric oxide signaling plays an important role in both conduit and resistant arteries in the environment of hypertension. Of note, the mean blood pressure is 20 mmHg higher in the eNOS knockout mice compared to their wild-type littermates [17]. Therefore, understanding the molecular mechanisms underlying impaired nitric oxide bioavailability and eNOS dysfunction in hypertension may prove beneficial in ultimately promoting development of novel therapeutics to treat hypertension.

Several mechanisms have been found responsible for nitric oxide deficiency in hypertension [9,18–24]. Destruction of nitric oxide by superoxide anion leads to nitric oxide deficiency, endothelial dysfunction, and high blood pressure. Among many enzymatic systems producing reactive oxygen species (ROS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase, uncoupled eNOS, mitochondria, and cyclooxygenase (COX) have been extensively studied [18,25]. Here, we take COX for example, and the other ROS systems will be described in the later sections below. During the generation of prostanoids by COX, ROS are formed as by-products [26]. In most tissues, COX-1 is constitutively expressed, whereas COX-2 is often induced by a number of inflammation or growth factors [27]. In small resistance arteries of essential hypertensive patients, COX-2 is overexpressed and reduces nitric oxide availability, and COX-2 represents a major source of oxidative stress generation [28]. Indeed, endothelium-dependent contractions were triggered by acetylcholine (ACh) after inhibition of NO production and they were abolished by COX-2 but not COX-1 inhibitors [29]. In addition, COX-2-derived prostaglandin F(2α) plays an important role in mediating endothelial dysfunction in renovascular hypertension [30]. Recently, Liu *et al.* [31] identified that uncoupling protein 2 inhibited oxidative stress and downregulated COX-2 expression to prevent endothelial dysfunction. ROS from one source are able to trigger ROS production by activating other enzyme systems [32–34]. For example, ROS produced by NOX can up-regulate the expression of COX-2 by p38 mitogen-activated protein kinase (MAPK)-dependent mechanism [32,34]. Oxidation of the eNOS cofactor tetrahydrobiopterin by peroxynitrite, a product of nitric oxide/superoxide interaction, induces eNOS uncoupling to produce superoxide rather than nitric oxide, further sustaining oxidative

stress (see 'Reactive oxygen species and endothelial nitric oxide synthase uncoupling' section).

Moreover, a defective L-arginine/nitric oxide pathway has been linked to nitric oxide deficiency in hypertension. Recent studies have confirmed that L-arginine transport is impaired in hypertensive and normotensive patients with a genetic background of essential hypertension [20], and the offspring of essential hypertensive patients are characterized by a reduced response to ACh linked to a defect in the nitric oxide pathway [19]. These data represent the link between L-arginine and the onset of essential hypertension. Furthermore, it has been shown that L-arginine supplementation improved endothelial dysfunction in hypertension [35]. The K_m of eNOS for L-arginine is about 3 μmol/l, but the concentration of plasma L-arginine rarely falls below 60 μmol/l in pathological conditions [36]. An elevation in asymmetric dimethylarginine (ADMA) levels may explain this 'L-arginine paradox', since ADMA is an endogenous competitive inhibitor of NOS [37]. Oxidative stress-dependent increase in circulating ADMA could lead to eNOS uncoupling [38], vasoconstriction [39], and therefore a marked increase in blood pressure [40,41]. Of note, elevated ADMA levels have been observed in hypertension, hypercholesterolemia, diabetes, and chronic kidney failure (CKD) [42]. Because ADMA is normally cleared by the kidney, patients with CKD have more than 3–10-fold higher plasma ADMA levels, which would lead to eNOS dysfunction and hypertension [43,44]. Regulation of eNOS function by tetrahydrobiopterin (H_4B) deficiency will be discussed in the section below entitled 'Consequences/contributions of eNOS uncoupling in hypertension'.

MECHANISMS OF ENDOTHELIAL NITRIC OXIDE SYNTHASE DYSFUNCTION

Synthesis of nitric oxide by nitric oxide synthase

Three NOS isoforms, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), catalyze the reaction of molecular oxygen with the amino acid substrate L-arginine to produce L-citrulline and nitric oxide [40,45,46]. nNOS is expressed in the central and the peripheral nervous system, and produces nitric oxide that functions as a neurotransmitter [47]. It has been shown that nNOS is compensatorily upregulated in hypertension [48]. Different from constitutively expressed nNOS and eNOS, the expression of iNOS is markedly increased by inflammatory cytokines [49]. Large quantities of nitric oxide produced by iNOS induce apoptosis to serve as a host defense mechanism, but also contribute to pathogenesis of chronic diseases [50]. eNOS, is constitutively expressed in vascular endothelial cells, and primarily located in the peri-nucleus, Golgi apparatus, and caveolae [51,52]. The NOS enzymes require several cofactors for enzymatic production of nitric oxide, including H_4B, NADPH, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) [53]. During the process of nitric oxide synthesis, the electrons donated by NADPH at the C-terminal reductase domain are transferred to the heme catalytic center of the N-terminal oxidase domain, where activation of molecular oxygen is 'coupled' to nitric oxide synthesis by two successive monooxygenations of L-arginine [46,54,55].

Reactive oxygen species and endothelial nitric oxide synthase uncoupling

Under some disease conditions, NOX activation increases the local production of ROS, which oxidizes H_4B to induce H_4B deficiency [56]. H_4B promotes assembly of eNOS monomers into an active dimer, whereas lack of H_4B results in inability of electron transfer to the N-terminal oxygenase domain of the other eNOS monomer [57,58]. Treatment of endothelial cells with peroxynitrite led to reduced eNOS activity and disruption of eNOS dimers [59]. Peroxynitrite is believed to be primarily responsible for oxidation of H_4B to dihydrobiopterin (H_2B) *in vivo*, although H_4B is sensitive to oxidation from many other ROS species [60,61]. The oxidized form H_2B can compete with H_4B for eNOS interaction, and they share the same binding sites in the N-terminal oxygenase domain of eNOS [62]. However, H_2B cannot support eNOS cofactor activity, so this displacement results in eNOS uncoupling. Therefore, under oxidative stress conditions, the salvage pathway of H_4B synthesis will become particularly important. Expanded discussions of mechanisms and contributions of eNOS uncoupling in hypertension are included in 'Role of endothelial nitric oxide synthase uncoupling in angiotensin II-dependent hypertension' and 'Role of endothelial nitric oxide synthase uncoupling in low renin, deoxycorticosterone acetate-salt hypertension' sections below.

Phosphorylation regulation of endothelial nitric oxide synthase function

Regulation of eNOS function through phosphorylation has been well established. The activity is regulated through phosphorylation or dephosphorylation [63–66]. Whereas phosphorylation of Ser615, 633, and 1177 results in the activation of eNOS, the phosphorylation of Thr495 reduces eNOS function [67]. Among these phosphorylated sites, Ser1177 and Thr495 are mostly investigated. The kinases taking part in eNOS phosphorylation at Ser1177 include 5' adenosine monophosphate-activated protein kinase (AMPK), protein kinase B (or Akt), extracellular signal-regulated kinases 1/2 (ERK1/2), and calcium/calmodulin-dependent kinase II (CaMK II) [68–71]. Reduced phosphorylation level at this site has been found in various forms of cardiovascular diseases, such as portal hypertension, diabetes, atherosclerosis, and myocardial infarction [67]. Of note, there seems to be a dynamic regulation of Ser1177 by transient hydrogen peroxide, acutely with ERK and chronically with Akt [33,69,71]. Moreover, Akt appears to be downstream of AMPK in this pathway [69]. In addition, a deficiency in Akt/eNOS signaling indicates impaired insulin signaling in type 2 diabetes. In aortas isolated from diabetic animals and in type 2 diabetic patients, Akt and eNOS phosphorylation was decreased [72,73]. Reduced $eNOS_{s1177}$ phosphorylation, vascular dysfunction, and elevated blood pressure have been observed in high-fat-fed animals [74]. On the contrary, AMPK and protein kinase C (PKC) can phosphorylate eNOS at Thr495, and its increased

phosphorylation may contribute to hypoxia, diabetes, and cerebral ischaemia [67]. Modulation of eNOS activity by dynamic changes in its phosphorylation level at different phosphorylation sites appears to be an important mechanism of eNOS regulation under different pathophysiological conditions.

Other factors and endothelial nitric oxide synthase dysfunction

In addition to L-arginine deficiency discussed earlier, several other mechanisms have been implicated in causing eNOS dysfunction/uncoupling. These include acetylation of eNOS, S-glutathionylation of eNOS, and protein–protein interactions other than phosphorylations discussed above. Cigarette smoking-induced oxidative stress downregulates silent information regulator protein 1 (SIRT1), leading to acetylation of eNOS and reduced nitric oxide production [75]. In addition, eNOS can also be acetylated by histone deacetylase 3 (HDAC3), which decreases nitric oxide production by reduced calmodulin association [76]. More recently, S-glutathionylation of eNOS has been proposed as an alternative mechanism provoking eNOS uncoupling [77]. Its inter-relationship with H_4B deficiency, however, remains to be further investigated. Two highly conserved systeine residues, Cys 689 and Cys 908, in the C-terminal reductase domain have been identified as sites of S-glutathionylation, which are important for eNOS function. Of note, eNOS S-glutathionylation in endothelial cells, accompanied by loss of nitric oxide and gain of superoxide, is associated with impaired endothelium-dependent vasodilation [77,78]. Finally, the activity of eNOS can also be regulated by binding to its regulatory binding partners such as calmodulin (CaM), caveolin-1 (Cav-1), heat shock protein 90 (HSP90), and NOS-interacting protein (NOSIP). For example, the association with Cav-1 inhibits eNOS activity and reduces nitric oxide production [79]. In Cav-1 knockout mice, both eNOS activity and vasorelaxation are enhanced in blood vessels [80]. In addition, Cav-1 deficiency was associated with attenuated Ang II-induced hypertension by inhibiting AT1a receptor-mediated uptake of Ang II in the renal proximal tubule [81].

CONSEQUENCES/CONTRIBUTIONS OF ENDOTHELIAL NITRIC OXIDE SYNTHASE UNCOUPLING IN HYPERTENSION

Role of endothelial nitric oxide synthase uncoupling in angiotensin II-dependent hypertension

Angiotensin II (Ang II) levels are often elevated in the kidney and plasma of patients with hypertension [82,83]. AT1 receptor antagonists effectively reduce blood pressure in Ang II-dependent hypertension [84,85]. Our previous study has confirmed that eNOS uncoupling contributes to high blood pressure in Ang II-infused mice, where aortic nitric oxide production was markedly decreased [86]. Dihydrofolate reductase (DHFR) overexpression or folic acid restoration of DHFR function effectively recoupled eNOS to reduce blood pressure [86,87]. Moreover, in Ang II-infused hyperphenylalaninemia (hph)-1 mice, where 79% of the animals developed severe abdominal aortic aneurysm (AAA), oral folic acid administration completely prevented AAA from occurring [87]. eNOS uncoupling-mediated aneurysm formation was also prevented by folic acid in Ang II-infused apoE null mice, and again this was attributed to targeted restoration of endothelial DHFR expression and activity [88]. The molecular mechanisms of Ang II-induced eNOS uncoupling have been characterized by our group, which involves a rapid and transient activation of endothelial NOX, subsequent H_2O_2-dependent down-regulation of DHFR, and persistent H_4B deficiency (Fig. 1) [33,56,89]. More specifically, the NOX isoform 1 (NOX1) has been identified as the mediator of Ang II-dependent uncoupling of eNOS in streptozotocin (STZ)-induced diabetic mice [56,89].

Dihydrofolate reductase is the rate-limiting salvage enzyme of H_4B, and is responsible for maintaining normal H_4B bioavailability by regeneration of H_4B from its oxidized form H_2B [90]. Therefore, impaired DHFR function is anticipated to lead to eNOS dysfunction [33]. Indeed, several studies have confirmed the crucial role of DHFR in maintaining H_4B and nitric oxide bioavailability, and hence the coupling state of eNOS [33,86,91]. RNAi inhibition of DHFR expression increased eNOS-dependent superoxide production, which was accompanied by reduced nitric oxide bioavailability, implying uncoupling of eNOS [33,91]. Moreover, in angiotensin-converting enzyme (ACE) knockout mice, where Ang II production was diminished, aortic DHFR protein abundance was significantly upregulated [33]. All of these findings suggest that DHFR is critically involved in preserving eNOS coupling and blood pressure in the model of Ang II-induced hypertension. Of note, additional studies have also confirmed an important role of DHFR in regulating eNOS coupling/uncoupling activity and vascular function. Crabtree *et al.* [91] showed that DHFR takes part in controlling H_4B/H_2B ratio, which is different from the function of guanosine triphosphate (GTP) cyclohydrolase I (GTPCH I) that regulates total biopterin levels. Although either DHFR or GTPCH I knockdown reduced VEGF-dependent nitric oxide production, only DHFR RNAi led to formation of ROS [92], implying its role in preserving eNOS coupling activity. Moreover, DHFR expression was found to be downregulated in an Ang II-dependent fashion during renal ischemia [93], which is similar to our findings in Ang II-infused hypertensive mice [86]. Furthermore, DHFR expression was decreased in 6 and 12 months old LDLR−/− animals, corresponding to impaired endothelial function [94].

Role of endothelial nitric oxide synthase uncoupling in low renin, deoxycorticosterone acetate-salt hypertension

The association between a high salt intake and hypertension has been investigated for a long time [95]. An increase in dietary salt leads to increased arterial blood pressure in individuals with salt-sensitive hypertension [96]. One commonly used model, namely DOCA-salt hypertension, was firstly established by Selye and colleagues in 1943 [97]. Young rats were co-treated by DOCA and 1% NaCl solution for 7 weeks, and the mean blood pressure (MBP) increased

to 187/130 mmHg, compared to 110/80 mmHg in the sham controls [97]. Previous studies have confirmed that NOX is the initial source of ROS leading to H_4B oxidation. H_4B treatment attenuated eNOS uncoupling, and blunted the blood pressure increase in DOCA-salt-induced hypertension [98]. In addition, mitochondria may also contribute to endothelin 1 (ET-1)-dependent oxidative stress in DOCA-salt rats [99]. Recent studies revealed an endothelial sepiapterin reductase (SPR) deficiency in aortic endothelial cells from DOCA-salt-hypertensive mice [100]. SPR takes part in modulating H_4B biosynthesis in both de-novo synthetic pathway and salvage pathway, implicating its indispensable role in regulating nitric oxide bioavailability [101]. Interestingly, SPR overexpression increased H_4B content, nitric oxide production, and nitric oxide-dependent vasorelaxation in both cultured cells and mouse models. RNAi of SPR had opposite effects [102]. Because SPR was lost in the endothelium of DOCA-salt-induced hypertensive mice, supplementation of sepiapterin, which needs to be metabolized to H_2B by SPR before its conversion to H_4B, had no effect on recoupling of eNOS. Nonetheless, combined administration of H_4B and a NOX inhibitor apocynin fully restored nitric oxide bioavailability [100]. On a separate note, overexpression of the H_4B synthetic enzyme GTP hydrocyclolase I (GTPCH I) was partially effective in improving endothelial function in DOCA-salt-hypertensive rats [103]. This partial effect may be explainable by the SPR deficiency that prevents maximal biosynthesis of H_4B in the presence of overexpressed GTPCH I.

POTENTIAL NEW THERAPIES TARGETING UNCOUPLED ENDOTHELIAL NITRIC OXIDE SYNTHASE IN HYPERTENSION

Given that eNOS uncoupling is one of the central pathogenic mechanisms of hypertension, restoration of adequate nitric oxide signaling via restoration of eNOS coupling activity in the blood vessels may serve as an important therapeutic strategy for hypertension. Restoration of cofactor bioavailability and inhibition of upstream pathways could represent promising strategies to recouple eNOS from its uncoupled state.

Restoration of cofactor bioavailability

Tetrahydrobiopterin supplementation has a great therapeutic potential of improving endothelial dysfunction in hypertension [46]. It augments endothelium-dependent vasodilation in both normotensive and hypertensive patients [22]. Basic experimental data from cultured cells and animal models support its efficacy in recoupling eNOS [92]. In addition, ascorbate (vitamin C) is important in maintaining H_4B levels in the setting of vascular oxidative stress [104], and treatment of bovine aortic endothelial cells (BAECs) with both H_4B and ascorbate prevented uncoupling of eNOS by $ONOO^-$ [105]. There are some evidences demonstrating that ascorbate improved endothelial function through regulation of eNOS in a genetic model of hypertension [106], which is mediated by increased H_4B stability and its intracellular amount [107,108]. Moreover, H_4B has been used in various experimental models. In spontaneously hypertensive rats (SHRs), H_4B supplementation diminished eNOS-dependent generation of ROS, while increasing nitric oxide production [109]. Oral administration of H_4B reduced vascular ROS production, increased nitric oxide production detected by electron spin resonance (ESR), and blunted the increase in blood pressure in DOCA-salt hypertension [98]. However, there is a limitation in scope for the potential clinical use of H_4B as a pharmaceutical drug, largely due to its chemical instability. H_4B can be easily oxidized to 7, 8-H_2B. Nevertheless, sapropterin dihydrochloride (6R-H_4B) is a novel thermo and photostable H_4B derivate that is commercially available for use as a phenylketonuria drug [110].

In addition, sepiapterin administration may be considered as another option to supply H_4B. Sepiapterin is firstly metabolized to H_2B by SPR and further to H_4B by DHFR [32]. Sepiapterin supplementation has been employed to recouple eNOS in cell culture and animal models. Treatment of BAECs with sepiapterin improved H_4B and nitric oxide bioavailabilities [102]. Furthermore, administration of sepiapterin markedly improved endothelium-dependent vasodilatation to different agonists [111]. All these data demonstrate that sepiapterin administration has the potential to be developed as an alternative treatment for hypertension.

Inhibition of upstream pathways

Since NOX has been identified as an initial activator to uncouple eNOS in both Ang II-dependent [86] and DOCA-salt-induced hypertension [98,100], it can be considered as a logical target for drug development for hypertension. Two isoforms, NOX1 and NOX2, have been shown to play crucial roles in hypertension, by promoting uncoupling of eNOS. The Ang II-induced increase in blood pressure was found reduced in mice deficient in NOX1 [112], NOX2 [113], or their catalytic subunit p47phox [114]. Of note, p47phox has been shown to interact with NOX1 as well, in addition to well received notion of interacting with NOX2 [56,115]. The impaired endothelial function in DOCA-salt hypertension was abolished in p47phox knockout mice [98]. Therefore, targeting the subunit or isoforms selectively and specifically might be beneficial to prevent or treat hypertension. The widely used NOX inhibitors include apocynin, an inhibitor of translocation of p47phox; statins, indirect inhibitors of cytosolic activator of NOX Rac1 [116], and Nox2ds-tat [117]. Recently, several inhibitors have been developed and found to be NOX-specific [118]. VAS2870 and its derivative VAS3947 function as pan-NOX inhibitors [119,120]. VAS2870 was found to inhibit NOX activity in smooth muscle cells [121] and human umbilical vein endothelial cells [122]. Impaired ACh-induced relaxation in SHR aortas was significantly attenuated by VAS2870 [120]. VAS3947 displays an improved solubility compared to VAS2870. It attenuated NOX activity completely in SHR aortas, without affecting either NOS activity or xanthine oxidase activity in phorbol 12-myristate 13-acetate (PMA)-stimulated human promyelocytic leukemia cells [119].

Recent studies have identified GKT137831 as a specific inhibitor for NOX1 and NOX4 [123]. In human aortic endothelial cells, high glucose-induced NOX1 activation was

TABLE 1. Mechanisms regulatory of endothelial nitric oxide synthase function

Post-translational regulation of eNOS	References
Uncoupling of eNOS	
H_4B deficiency	
Oxidation by $ONOO^-$	[60]
Deficiency of GTPCH I	[127,128]
Deficiency of SPR	[100,102]
Deficiency of DHFR	[86,91,129]
Deficiency of L-arginine	[130,131]
Disruption of dimerization	[59]
Protein modification	
Phosphorylation	
Phosphorylation at T495	[65,66,132–134]
Dephosphorylation at S1177	[65,133–135]
Acetylation	
Downregulation of SIRT1	[75,136]
Inhibition of HDAC3	[76]
S-glutathionylation[a]	[77]
Protein–protein interaction	
Dissociation with HSP90	[137–139]
Binding to caveolin-1	[80,138,140]

DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; GTPCH I, GTP cyclohydrolase 1; H_4B, tetrahydrobiopterin; HDAC3, histone deacetylase 3; SIRT1, silent information regulator protein 1; SPR, sepiapterin reductase.
[a]S-glutathionylation uncouples eNOS.

attenuated by GKT137831 and by NOX1 RNAi. In addition, administration of GKT137831 significantly attenuated lesion formation in diabetic apolipoprotein E-deficient mice, which was comparable to that seen in NOX1/apoE double knockout mice [123]. ML171 is a compound that was identified by cell-based high-throughput screening, employing ROS-detecting chemiluminescence in NOX1-overexpressing cells [124]. This inhibitor blocked the formation of functional invadopodia in human colon cancer cells, which is well established to be a NOX1-specific response [124]. Fulvene-5 efficiently inhibited NOX activity measured by hydrogen peroxide in 293 cells stably transfected with constitutively active NOX4, and COSphox cells harboring inducible Nox2/p47phox/p67phox complex, showing that fluvene-5 can act as a NOX2 and NOX4 inhibitor [125]. Indeed NOX4-induced ROS production and arrhythmic phenotype in zebrafish was abolished by fluvene-5 and 6-dimethylamino fluvene [126]. However, none of these NOX inhibitors has been tested in patients with hypertension, although they are highly promising drug candidates for hypertension via preservation of eNOS coupling activity. Given that Ang II is a potent NOX activator for subsequent induction of eNOS uncoupling, attenuation of Ang II signaling is clearly another feasible strategy to inhibit eNOS uncoupling in hypertension.

In conclusion, this review gives an overview of a dynamic research field of how eNOS uncoupling contributes to hypertension, and how eNOS recoupling may serve as a novel and effective therapeutic strategy for hypertension. The major regulatory mechanisms of eNOS are listed in Table 1. Supplementation of H_4B, its stable alternatives or precursor sepiapterin, or inhibition of NOX, may represent promising new therapeutics to preserve eNOS coupling activity for the treatment of hypertension.

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Conflicts of interest

The authors have no conflicts of interest to report.

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