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FORUM REVIEW ARTICLE

Oxidative Stress, GTPCH1, and Endothelial Nitric Oxide Synthase Uncoupling in Hypertension

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Abstract

Significance: Hypertension has major health consequences, which is associated with endothelial dysfunction. Endothelial nitric oxide synthase (eNOS)-produced nitric oxide (NO) signaling in the vasculature plays an important role in maintaining vascular homeostasis. Considering the importance of NO system, this review aims to provide a brief overview of the biochemistry of members of NO signaling, including GTPCH1 [guanosine 5′-triphosphate (GTP) cyclohydrolase 1], tetrahydrobiopterin (BH_4), and eNOS.
Recent Advances: Being NO signaling activators and regulators of eNOS signaling, BH_4 treatment is getting widespread attention either as potential therapeutic agents or as preventive agents. Recent clinical trials also support that BH_4 treatment could be considered a promising therapeutic in hypertension.
Critical Issues: Under conditions of BH_4 depletion, eNOS-generated superoxides trigger pathological events. Abnormalities in NO availability and BH_4 deficiency lead to disturbed redox regulation causing pathological events. This disturbed signaling influences the development of systemic hypertension as well as pulmonary hypertension.
Future Directions: Considering the importance of BH_4 and NO to improve the translational significance, it is essential to continue research on this field to manipulate BH_4 to increase the efficacy for treating hypertension. Thus, this review also examines the current state of knowledge on the effects of eNOS activators on preclinical models and humans to utilize this information for potential therapy. *Antioxid. Redox Signal.* 34, 750–764.

Keywords: hypertension, eNOS uncoupling, GTPCH1, endothelial nitric oxide synthase

Background

HIGH BLOOD PRESSURE (BP) epidemic is a major public health concern and most patients will require pharmacologic interventions to control their BP. The role of nitric oxide (NO) has its importance in keeping up vascular health as well as for the control of BP. The very important members of the NO system include guanosine 5′-triphosphate (GTP)-cyclohydrolase 1 (GTPCH1) and nitric oxide synthases (NOS), mainly endothelial NOS (eNOS) and some essential cofactors: tetrahydrobiopterin (BH_4), calmodulin, heme, flavins, and NADPH. Thus, BP risk management by improving endothelial bioavailability of NO had emerged as a major research goal by many research groups. However, the importance of NO had been highlighted by many key observations in animal models and humans in the past several decades; a lack of understanding still exists as a gap that is supposed to be filled up. This is evidenced by a few clinical trials that were done before to improve the NO bioavailability to control BP. Although these trails were ideally expected to have a positive effect on controlling BP, it ended up with less clinical significance. Therefore, compiling conclusive evidence on experimental, preclinical, and clinical reports on NO signaling and its importance in BP homeostasis is important.

The review is organized as follows. It starts with describing the relationship between the three important members of the NO system, including eNOS, BH_4, GTPCH1 and the

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damping factors of NO bioavailability, mainly, eNOS uncoupling and oxidative stress. Furthermore, the review briefly describes the potential NO signaling modulators and their clinical perspectives.

Hypertension Epidemiology

Hypertension, otherwise known as a condition of elevated BP—is a serious medical condition that significantly increases the risks of myocardial infarction, stroke, cardiac failure, and renal failure if not controlled (45). An estimated 1.13 billion people worldwide have hypertension (120), and it is one of the leading risk factors, reason for all-cause mortality that kills 9 million people each year. As two-thirds of the people with hypertension are living in low- and middle-income countries (120), hypertension could be considered a socioeconomic burden for these countries. The trend of the highest BP level during these past four decades has shifted from high-income countries to low-income countries. Contrastingly, BP has been persistently high in Central and Eastern Europe (23, 76), supporting increased death rates from stroke (98). Given its high burden and the aging of the population, hypertension remains an issue of global concern (12). However, many impressive progressions were made over several decades to improve hypertension detection and treatment, still the strategy to develop primary prevention is a subject of growing interest (67).

Nitric Oxide

Free radical NO is an unorthodox messenger molecule that is gaseous and lipophilic. It is one of the most important signaling molecules in maintaining vascular homeostasis. NO is synthesized from the oxidation and catalytic conversion of L-arginine to citrulline in the presence of molecular oxygen and different cofactors such as BH_4, NADPH, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Its concentration within the biological system is regulated by the activity of a family of NOS enzymes (90, 92). Besides, NOS isoform NO can also be produced by xanthine oxidoreductase or cytochrome P450 reductase. Besides, NO can be released nonenzymatically from iron-nitrosyl hemoglobin, S-nitrosylated blood proteins (21). Through its cyclic guanosine monophosphate (cGMP)-dependent mechanisms, NO exerts a wide variety of biological functions but not limited to maintaining homeostasis, including modulation of vascular tone, vasodilation, vascular permeability, antiplatelet, antithrombotic, and anti-inflammatory properties within the vasculature (15). Furthermore, NO produced by eNOS in the endothelium diffuses to vascular smooth muscle cells and activates the soluble guanylyl cyclase-cGMP-dependent protein kinase pathway, eventually leading to vasodilation (81).

NO is generated by NOS in nearly all types of mammalian cells. Chemically, NO is very unstable and is highly reactive with reactive oxygen species (ROS) such as superoxide anions ($O_2\cdot^-$). Thus, the amount of NO is not only dependent upon its production by NOS but also by the rate of its inactivation by ROS, including $O_2\cdot^-$. NO also undergoes rapid radical/radical or oxidation reactions to produce a plethora of biologically active derivatives. The other free radicals produced by various reactions with free NO are collectively called as reactive nitrogen species (RNS), which also play crucial roles in the maintenance of physiological homeostasis. RNS include NO radicals and related species, including nitric oxide ($NO\cdot$), nitrogen dioxide ($NO_2\cdot$), nitrosyl (NO^+), nitroxide (NO^-), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), nitronium ion (NO_2^+), peroxynitrite ($ONOO^-$), nitroso persulfide ($SSNO^-$), and alkyl peroxynitrites (RONOO) (92). These RNS can further produce nitrosothiols, nitrosamine, nitro fatty acids, and so on (66).

eNOS regulation, activation, and eNOS uncoupling

Physiologically important NO is produced by eNOS, which is an important regulator of vascular function. The human eNOS gene is located on chromosome 7q35–36, consisting of 26 exons spanning ~21 kb with multiple polymorphisms that might confer risk for hypertension. An association between eNOS polymorphism and reduced eNOS expression and activity, as well as increased oxidative stress in the vascular endothelium, has been reported by many research groups (60).

Many kinases were reported to regulate eNOS phosphorylation and NO production, under various stimuli. These kinases include Akt/PKB, cAMP-dependent protein kinase (PKA), and the AMP-activated protein kinase (AMPK). Importantly, AMPK, Akt, ERK1/2, and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) activated Ser1177 phosphorylation, enhancing the specific activity of eNOS, so that NO production is boosted in the absence of a maintained increase in intracellular Ca^{2+}. The other known eNOS phosphorylation that results in activation of eNOS is Ser615 and 633, and the inhibitory phosphorylation is Thr-495. Under the stimulation of vascular endothelial growth factor (VEGF) in endothelial cells, Ser1177 phosphorylation is activated, while Thr-495 phosphorylation is transiently reversed (Fig. 1) (72).

The promoter region in the eNOS gene encodes transcription of eNOS protein, which plays a critical role in the regulation of eNOS expression. The human NOS3 promoter contains two regulatory regions, positive regulatory domains I and II. A positive regulatory domain I links to a high-affinity Sp1 transcription factor recognition site and binds to three nucleoproteins identified as Sp1 and two variants of Sp3. The positive regulatory domain II forms nucleoprotein complexes with the positively regulating transcription factors Ets-1, Elf-1, YY1, and Sp1, and the inhibitory factor MYC-associated zinc finger protein (58, 87).

The catalytic mechanisms of NOS involve flavin-mediated electron transport from the C-terminal-bound NADPH to the N-terminal heme center where oxygen is reduced and incorporated into the guanidine group of L-arginine giving rise to NO and L-citrulline. Thus, NOS activity requires FMN, FAD, BH_4, Ca^{2+}-calmodulin, and heme (37). Another critical determinant of eNOS activity is the availability of BH_4 (3). eNOS must be fully saturated with BH_4 to completely couple NADPH oxidation to NO production (Fig. 1). Under conditions of limited BH_4 availability, eNOS functions in an ''uncoupled'' state in which NAD(P)H-derived electrons are added to molecular oxygen rather than L-arginine, thereby resulting in the formation of $O_2\cdot^-$, instead of NO (89, 91). Therefore, its chemical environment (*i.e.*, presence of superoxide) determines whether NO exerts protective or harmful effects by producing ROS or RNS. In this setting, uncoupled eNOS exacerbates oxidative stress that is initiated by other ROS-generating enzymes (*e.g.*, NADPH oxidase, NOX). This phenomenon is referred to as NOS ''uncoupling'' and was first

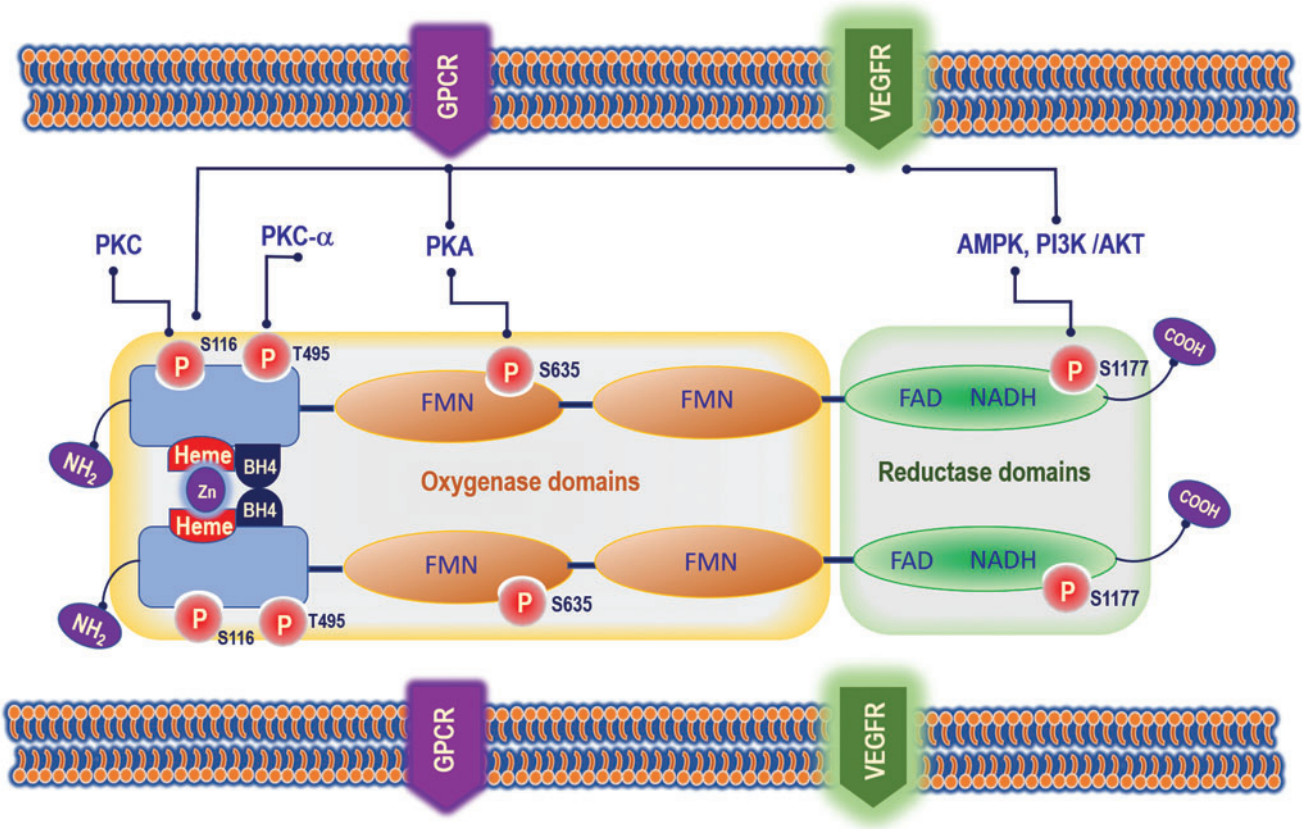

FIG. 1. Mechanisms by which eNOS activity is regulated in endothelial cells. eNOS is regulated by PKC (phosphorylates at S116); PKC-α (phosphorylates at T495); PKA (phosphorylates at S635); and AMPK, PI3K/AKT (phosphorylates at S1177). eNOS is an obligate homodimer and this dimeric association is mediated by a cysteine-complexed Zn^{2+} (zinc-tetrathiolate) at the dimer interface. AMPK, 5′ adenosine monophosphate-activated protein kinase; BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NO, nitric oxide. Color images are available online.

demonstrated in purified neuronal NOS (nNOS) (51, 88), and also extended to eNOS (121, 125). eNOS uncoupling requires Ca^{2+}/calmodulin and heme and will be blocked by the specific NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) (121). $ONOO^-$ directly uncouples eNOS by oxidizing and releasing zinc from the zinc/thiolate cluster of eNOS and presumably forming disulfide bonds between the monomers (129).

L-NAME inhibits $ONOO^-$-mediated eNOS uncoupling. In addition, myeloperoxidase-derived hypochlorous acid (HOCl) also uncouples eNOS *via* $ONOO^-$-mediated oxidation of the zinc/thiolate center and zinc release (Fig. 2) (125). Thus, this transformation of eNOS makes it from a protective enzyme to a contributor to oxidative stress. Other biochemical mechanisms proposed to be involved in eNOS uncoupling are the increase of endogenous asymmetric dimethylarginine, L-arginine deficiency and oxidative stress (79). Besides, eNOS is regulated by a complex pattern of post-translational modifications. These modifications include S-nitrosation, glutathionylation, and persulfidation, which were reported to impact eNOS activity. In general, exogenous NO causes inhibition of eNOS activity in vascular endothelial cells. This is because exposure of NO free radicals or NO donor causes S-nitrosation of eNOS and diminishes its activity (93). The exogenous NO also can decrease

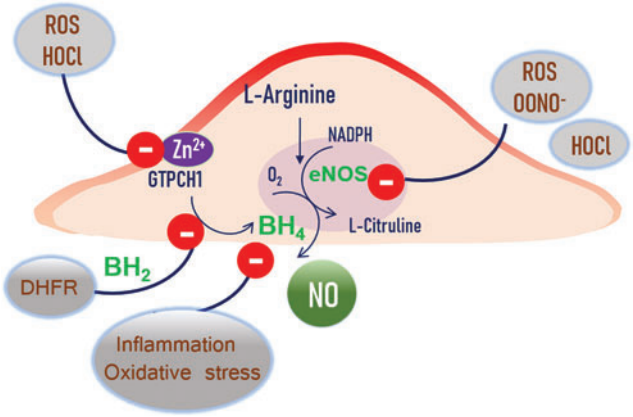

FIG. 2. Inhibitory factors of $NO^{\cdot}$ production. DHFR recycles the BH_4 to BH_2 and further inhibits the eNOS activity. ROS and HOCl target the GTPCH1 enzyme and release the Zn ion, and the BH_4 production is inhibited. Inflammation, oxidative stress, peroxynitrite, and HOCl all can target eNOS and reduce the $NO^{\cdot}$ production. GTPCH1, guanosine 5′-triphosphate (GTP)-cyclohydrolase 1; HOCl, hypochlorous acid; $O_2^{\cdot -}$, superoxide; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species. Color images are available online.

NOS activity along with decreasing of eNOS dimer levels. Whereas the presence of the thioredoxin and thioredoxin reductase system could prevent eNOS monomerization and loss of activity, indicating the inhibitory action of NO by disturbing the zinc tetrathiolate cluster at the dimeric interface through S-nitrosylation of the cysteine residues (93). Another study showed that in resting endothelial cells, eNOS is tonically S-nitrosylated and that the enzyme undergoes rapid transient denitrosylation after the addition of the eNOS agonist, VEGF (34). The receptor-mediated decrease in eNOS S-nitrosylation is inversely related to enzyme phosphorylation at Ser1179 (34). Another protein modification that can diminish the production of NO and enhance the superoxide generation is glutathionylation (20). Two highly conserved cysteine residues were identified as sites of S-glutathionylation in the reductase domain of eNOS and found to be critical for redox regulation of eNOS function. Protein thiols can undergo S-glutathionylation, which is considered to be a reversible protein modification that involves cellular signaling and adaptation. In eNOS, this S-glutathionylation of eNOS reversibly decreases NOS activity with an increase in $O_2\cdot^-$ and provides a pivotal switch providing redox regulation of cellular signaling, endothelial function, and vascular tone (20). DNA methylation in the eNOS promoter region is also involved in the cell-specific expression of the human eNOS gene (18), which allows the eNOS to be specifically expressed on endothelial cells. This methylation pattern exhibits a dramatic difference between endothelial and nonendothelial cell types, including vascular smooth muscle cells. Methylation exhibits a marked decrease in the synergistic action of Sp1, Sp3, and Ets1 on eNOS promoter activity and the addition of methyl-CpG-binding protein 2 further reduced the transcriptional activity of methylated eNOS constructs (18).

eNOS uncoupling has been implicated in a variety of experimental and clinical vascular disease states, including diabetes (114), hypertension (68), abdominal aortic aneurysms (43), and overt atherosclerosis (112). The role of eNOS uncoupling in vascular diseases is best exemplified by the fact that overexpression of eNOS in ApoE knockout (ApoE-KO/eNOS-Tg) mice does not inhibit, but accelerates atherosclerosis with increased $O_2\cdot^-$ production in aortic rings under hypercholesterolemia compared with ApoE-KO mice (112), suggesting that eNOS is uncoupled and contributes to atherogenesis. eNOS uncoupling also presents in hepatic ischemia/reperfusion injury in type 2 diabetic mice (32).

Role of NO in cardiovascular diseases

NO bioavailability indicates the production and utilization of endothelial NO in the vasculature. Importantly, decreased NO bioavailability exerts a critical role in hypertension development (53), a major risk factor for cardiovascular diseases. The endothelium is a central regulator of vascular tone and BP by virtue of its ability to produce NO (31, 110). Reciprocally, NO also clearly affects endothelial function. Endothelial dysfunction is an important risk factor for both hypertension and cardiovascular diseases. A hallmark of endothelial dysfunction is the loss of the protective actions of NO-NO bioavailability, due to a reduction in its synthesis by eNOS and an increase in scavenging by ROS (3, 33). Decreased NO bioavailability has been also widely described in vascular diseases such as atherosclerosis and disease factors, including cigarette smoking and diabetes.

There are three isoforms of NOS, eNOS (NOS III), nNOS (NOS I), and inducible NOS (iNOS, NOS II). The importance of eNOS-derived NO for BP regulation is supported by evidence of systemic hypertension in the eNOS knockout mice (57, 102) and hypotension in eNOS transgenic (eNOS-Tg) animals (86). It was demonstrated that nNOS-derived NO has an important role in the physiological regulation of BP in healthy humans (101). Moreover, in eNOS knockout mice, nNOS compensates eNOS-dependent vasodilation in coronary arteries (55), indicating the importance of NO in the vascular homeostasis. Similar to eNOS in central vascular beds, nNOS mediates vasodilation in peripheral and coronary vascular beds through NO production (99). An early feature of hypertension is abnormal endothelial function or endothelial dysfunction (13, 52). eNOS knockout mice have less NO production under physiological conditions compared with wild-type and iNOS knockout mice. Excessive NO production by iNOS induction results in hypotension and shock during sepsis (47). A single administration of the oral NO supplementation decreases BP and improves endothelial function in hypertensive patients (54). Thrombospondin-4 (Thbs4), which plays an important role in endothelial dysfunction, is involved in NO signaling. Palao *et al.* showed that Ang II induced impaired vasodilation in mesenteric arteries causing hypertension in WT mice, whereas in Thbs4 knockout mice, vasodilation was preserved indicating the role of Thbs4 in the NO-mediated signaling pathway and thereby in hypertension (87a). In another study, Lindsey *et al.* showed that G-1, an agonist of G protein-coupled estrogen receptor 1 (GPER), also known as G protein-coupled receptor 30, which induces vasorelaxation by inducing NO from endothelial cells through increasing cAMP signaling in smooth muscle cell (71). Besides, they also showed that GPER protects from AT II-induced hypertension thereby indicating the role of NO in GPER-mediated cardiovascular protection (85).

Mitochondria-associated production of NO

Several vascular diseases such as hypertension, ischemia/reperfusion injury, atherosclerosis, heart failure, cardiac hypertrophy, Alzheimer's disease, Parkinson disease (108), and diabetes (77, 78) are associated with mitochondrial dysfunction due to uncontrolled ROS (103). Thus, detecting an early stage of mitochondrial dysfunction might be a crucial step in treating cardiovascular diseases. Mitochondrial depolarization in endothelial cells activates eNOS leading to NO production by increased phosphorylation of eNOS through the PI3K/Akt pathway (60). Also, drugs such as BMS-191095 and diazoxide protect neurovascular components during ischemic/reperfusion injury through mitochondrial depolarization and NO production (61).

Aging is one of the main risk factors for cardiovascular diseases (48), and impaired mitochondrial respiration is observed in several cardiovascular diseases (96). Even though most of the studies have shown that NO produced by NOS isoforms inhibits mitochondrial respiration by binding with different complexes in mitochondrial respiration, the expression and function of NOS isoforms in mitochondria are controversial until recently (95). This is partly due to the lack of novel high-throughput techniques to measure respiration in isolated mitochondria to exclude the effect of extramitochondrial NOS effects. However, advancements in measuring mitochondrial respiration in isolated mitochondria

from various tissues such as the brain, heart, and kidney using extracellular flux assays (*e.g.*, Seahorse XFe96 analyzer) have enabled us to draw more conclusions about NOS isoforms in mitochondria (105). A recent study by Sakamuri *et al.* showed that a selective nNOS inhibitor ARL-17477 decreased mitochondrial respiration by decreasing S-nitrosylation of mitochondrial protein in freshly isolated mitochondria from both the brain and heart, whereas a selective eNOS inhibitor NIO [N5-(1-iminoethyl)-L-ornithine] has no effect on mitochondrial respiration indicating the role of NO in mitochondrial respiration, thereby in all cardiovascular diseases (95).

BH_4 Deficiency and eNOS Uncoupling in Hypertension

Cofactor BH_4 dose dependently inhibits recombinant eNOS uncoupling in the cell-free system. However, its substrate L-arginine cannot block eNOS uncoupling in the absence of BH_4 (121). BH_4 cannot be oxidized by $ONOO^-$ and cannot prevent eNOS oxidation and uncoupling by $ONOO^-$ (129). BH_4 is required for eNOS coupling *in vivo* even in the healthy state (11, 92, 104). Tissue levels of BH_4 reduce in disease conditions, including hypertension (30, 64, 128). BH_4 availability in the lung controls pulmonary vascular tone, right ventricular hypertrophy, and vascular structural remodeling in a dose-dependent manner under both normoxic and hypoxic conditions. Furthermore, BH_4 availability has striking effects on the immediate vasoconstriction response to acute hypoxia. BH_4 supplementation improves forearm blood flow in patients and blocks endothelial dysfunction in vessel rings from atherosclerosis (4).

Several studies have demonstrated that BH_4 deficiency is responsible for eNOS uncoupling in hypertension (Fig. 3). Landmesser *et al.* elegantly demonstrated that BH_4 reductions due to ROS-mediated BH_4 oxidation and eNOS uncoupling are evident in deoxycorticosterone acetate-salt (DOCA-salt) hypertensive mice. Oral BH_4 treatment dramatically reduces vascular ROS but increases NO production (eNOS recoupling), and consequently decreases BP in the DOCA-salt mouse model (64).

eNOS uncoupling triggered by BH_4 deficiency also presents in other disease states, since eNOS uncoupling is effectively prevented by coadministration of L-sepiapterin, a BH_4 precursor, or folic acid in diabetic animal models (32, 83), Ang II-AAA models (43), and in patients (106). These effects of BH4 are mediated through NO, but not $O_2\cdot^-$ synthesized by eNOS. Therefore, the presence of adequate BH_4 in the endothelium is critical for maintaining ''coupled'' eNOS in healthy subjects (11) and ''recoupling'' eNOS in patients with essential hypertension (Fig. 3) (36, 109). These findings support the concept that intracellular BH_4 concentrations dictate, at least in part, the balance of NO and $O_2\cdot^-$ produced by eNOS in healthy and diseased blood vessels.

BH_4 deficiency and pulmonary hypertension in the human population

Pulmonary hypertension (PH) is a hemodynamic and pathophysiologic state, which is a complex disease and associated with endothelial dysfunction. The PH condition may

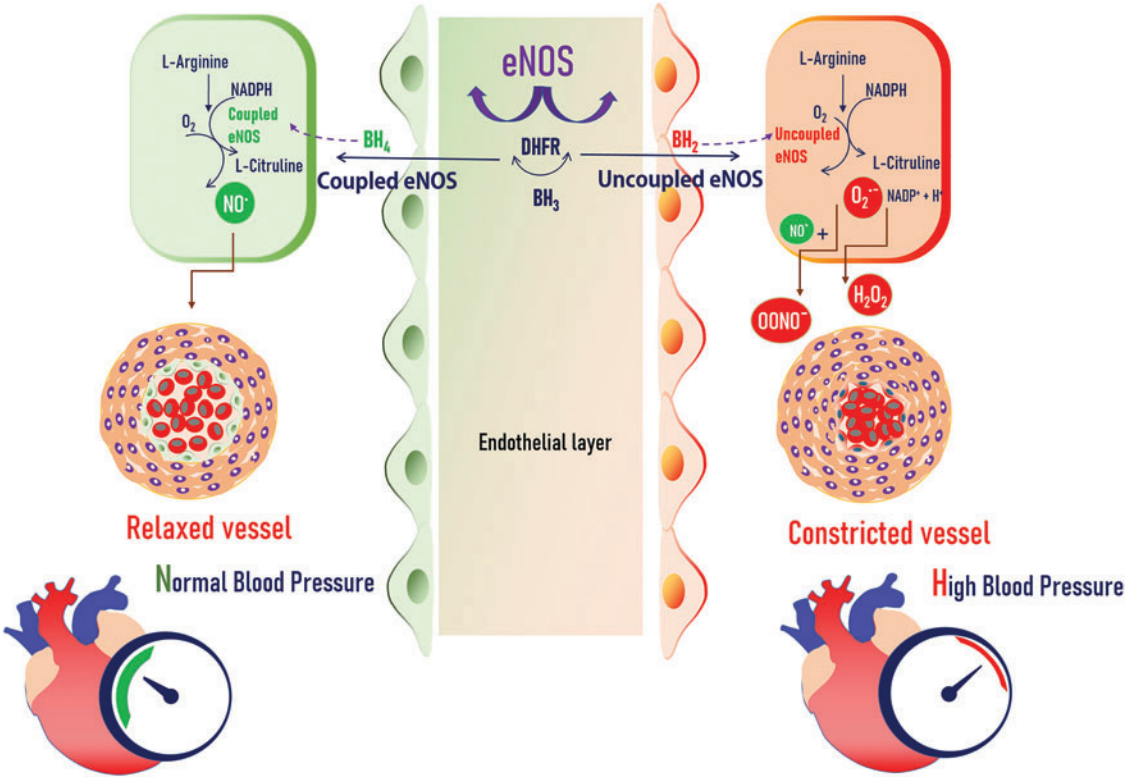

FIG. 3. Mechanisms by which coupled eNOS regulates the vascular homeostasis and uncoupled eNOS determines hypertension. Increased bioavailability of BH_4 and coupled form of eNOS lead to production of NO, by utilizing the L-arginine as a substrate. The $NO^{\cdot}$ further helps to keep the vessel homeostasis and normal blood pressure. Whereas the uncoupled form of eNOS along with increased BH2 levels helps vessel constriction and leads to high blood pressure. Color images are available online.

further trigger dyspnea, decreased exercise tolerance, and progression to right heart failure (8, 39). Similar to systemic hypertension, aberrant NO system and BH_4 deficiency have equal importance in developing PH. Pulmonary endothelium-produced NO has an important role in maintaining vascular homeostasis, and uncoupled eNOS has a pathogenetic role in cardiopulmonary disorders (44).

Chronic hypoxia-induced PH in the newborn pigs has shown evidence of eNOS uncoupling. To test rescuing and rebalancing the NO system in these models, the animals were treated either with L-citrulline or BH_4, or both together (29). Strategically, L-arginine is a precursor for L-citrulline and provides an alternate way to increase intracellular L-arginine. Study results showed that when compared with the untreated hypoxic group, pulmonary vascular resistance was lower in hypoxic piglets cotreated with L-citrulline and BH_4. Interestingly, these phenomena were not observed in hypoxic piglets treated with BH_4 alone. This indicates that the combination therapy of NO precursors and BH4 may offer enhanced therapeutic capacity to ameliorate PH (29). The *hph-1* (hyperphenylalaninemia 1) mice exhibit PH as they are deficient in the rate-limiting enzyme for BH_4 synthesis. They tend to develop PH phenotype under normoxic conditions without concomitant systemic hypertension (40). Thus, to study if BH_4 supplementation can rescue PH, Francis *et al.* utilized the MCT-induced PH rat model. By supplementing BH_4, preventive as well as rescuing experiments were done. The experimental results showed that BH_4 intervention restored normal levels of eNOS protein and ameliorated pulmonary vascular muscularization in a dose-dependent manner. Furthermore, BH_4 administration also reduced pulmonary artery pressure (40).

In humans, the patients with idiopathic pulmonary fibrosis (IPF), the pulmonary artery expression of eNOS is decreased in parallel to the increased iNOS and ROS as well as nitrotyrosine (97). In another study, BH_4 level in serum was found to be low in IPF patients, when compared with the control people. These patients also were found with the absence of GTPCH1 and eNOS expression in their pulmonary arteries (2). Thus, to understand if the intervention of the BH_4 could attenuate PH in IPF, the bleomycin-induced pulmonary fibrosis model was utilized. Similar to the human population, these animals also showed an absolute depletion of plasma BH_4 levels. The oral supplementation of sepiapterin (BH_4 analogue) rebalanced the BH_4 level and attenuated bleomycin-induced pulmonary fibrosis, mortality, vascular remodeling, and PH. Sepiapterin also inhibits the endothelial-to-mesenchymal transition induced by fibrotic mediators in pulmonary artery sections (2). The findings of these studies show that rebalancing BH_4 is essential to keep the pulmonary homeostasis and greater efficacy of oral BH_4 compound in ameliorating the development of PH.

GTPCH1, BH_4 Regulation, and Hypertension

BH_4 levels in endothelial cells are variable, and a continuous supply is required to maintain its basal levels (Fig. 2). In general, BH_4 levels are dictated by GTPCH1, dihydrofolate reductase (DHFR), the enzyme that recycles BH_4 in the salvage pathway (17, 26), and oxidative degradation of BH_4, namely BH_4 oxidation (Fig. 4) (64, 128). GTPCH1 is the first and rate-limiting enzyme in *de novo* BH_4 biosynthesis.

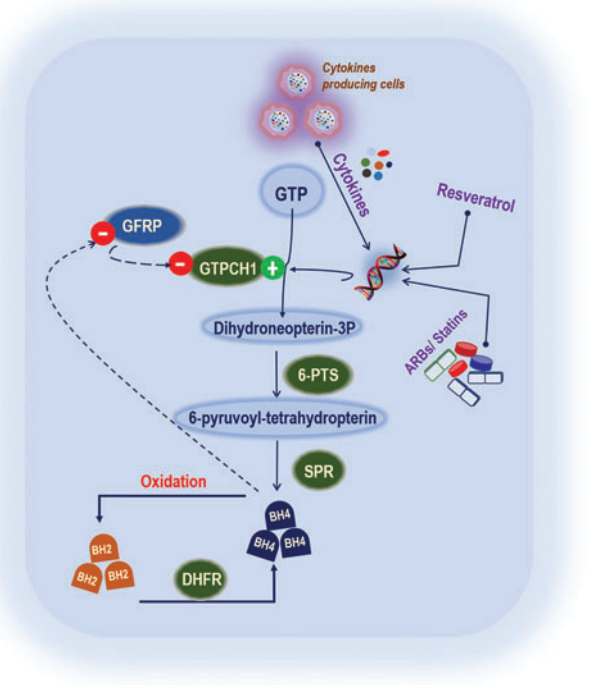

FIG. 4. GTPCH1 regulation and BH_4 synthesis. Cytokines, resveratrol, ARBs, and statins are known to increase the GTPCH1 expression as well as BH_4 synthesis. GTP is used as a substrate by GTPCH1 and produces dihydroneopterin-3P. Dihydroneopterin-3P is again used by 6-PTS and produces 6-pyruvoyl-tetrahydropterin and further converted into BH_4 by SPR. BH_2 is recycled by DHFR into BH_4, whereas BH_2 is produced by the oxidation of BH_4. 6-PTS, 6-pyruvoyl-tetrahydropterin synthase; ARBs, angiotensin receptor blockers; GFRP, GTP cyclohydrolase feedback regulatory protein; SPR, sepiapterin reductase. Color images are available online.

GTPCH1 catalyzes GTP to dihydroneopterin triphosphate (126). Dihydroneopterin-3P is again used by 6-pyruvoyl-tetrahydropterin synthase (6-PTS) and produces 6-pyruvoyl-tetrahydropterin and further converted into BH_4 by sepiapterin reductase SPR (Fig. 4). Thus, GTPCH1 is critical for the maintenance of BH_4 levels since its inhibition leads to a rapid BH_4 reduction. GTPCH1 is a homodecameric enzyme consisting of 25-kDa subunits in mammalian cells (117). Zinc ion generates a hydroxyl nucleophile for the attack of imidazole ring carbon atom eight of the substrate, GTP (Fig. 5).

Interestingly, the transfer of the GTPCH1 gene in cultured endothelial cells markedly increases BH_4 levels without altering the BH_4/BH_2 ratio *in vitro*, strongly suggesting that other enzymes in the BH_4 synthetic pathway do not become significantly rate limiting even when GTPCH1 is overexpressed (14). GTPCH1 overexpression reverses BH_4 deficiency and endothelial dysfunction in carotid arteries of DOCA-salt-induced hypertensive rats and mice *in vivo* (30, 128). GTPCH1 constitutively expresses in endothelial cells. In cultured human umbilical vein endothelial cells (HUVECs), acute stimulation with cytokines, interferon-γ plus tumor necrosis factor-α, increases GTPCH1 transcription *via* NF-κB/STAT1 pathways in

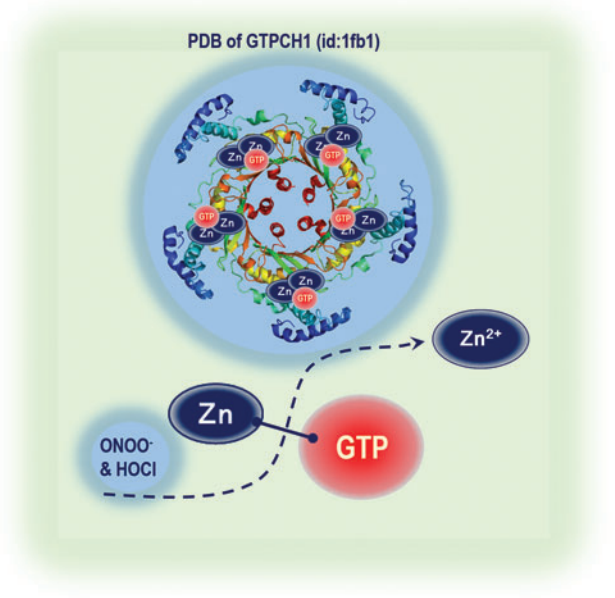

FIG. 5. GTPCH1 structure and zinc release by oxidants. GTPCH1 is a homodecameric enzyme. Zinc ion generates a hydroxyl nucleophile for the attack of imidazole ring carbon atom eight of the substrate, GTP. Both HOCl and $ONOO^-$ react fast with the positively charged zinc atom resulting in its release from zinc-containing proteins. Color images are available online.

a distinct but cooperative manner (50, 56). Lipopolysaccharide downregulates GTPCH1 feedback regulatory protein (GFRP) mRNA in HUVECs (119), which inhibits GTPCH1 (122). No information is available regarding the modulation of GTPCH1 activity by phosphorylation in vascular cells. These *in vitro* findings remain to be confirmed *in vivo* in vascular disease models. It is also worth noting that the upregulation of GTPCH1 may be a compensatory mechanism during early disease stages that eventually disappear as the disease progresses.

Several studies have shown that, in line with a BH_4 reduction and eNOS uncoupling, GTPCH1 activity or protein levels decrease in diabetes and hypertension. Endothelial cells isolated from diabetic BioBreeding rats showed reduced GTPCH1 levels (75), and this effect was reversed by GTPCH1 gene transfer (74, 124). Consistent with the findings, in the diabetes model, aortic GTPCH1 mRNA levels are reduced in a glucocorticoid-induced rat model of hypertension (80). However, whether this decrease is due to the glucocorticoid treatment is unclear (80). In the carotid arteries, GTPCH1 activity is decreased during the late stages of hypertension in DOCA-salt hypertensive rats (*i.e.*, after a 4-week treatment). Arterial gene transfer of human GTPCH1 restores GTPCH1 activity, restores BH_4 levels, and normalizes eNOS function in these animals (128). Conversely, *hph-1* mice exhibit significantly reduced systemic GTPCH1 expression and BH_4 synthesis (73). Initial studies of the vascular phenotype in this model revealed that endothelium-dependent NO-mediated relaxation is only minimally impaired in the aorta, despite a modest but significant increase in BP (24). These findings are consistent with the recent observations in DOCA-salt hypertensive mice that uncoupled eNOS produces H_2O_2 instead of NO (64), accounting for the minimally impaired relaxation in these conduit arteries (24, 64).

Because conduit arteries such as the aorta do not regulate total peripheral resistance, the hypertensive phenotype observed in *hph-1* mice (24) and the BP-lowering effect observed in BH_4-treated DOCA-salt mice (64) may be attributable to changes in the structure and function of resistance arteries (24, 64). However, more recent evidence indicates that BH_4 deficiency in DOCA-salt hypertensive mice is secondary to a reduction in GTPCH1 (62). Accordingly, BH_4 supplementation or increased BH_4 synthesis through adenoviral overexpression of *GTPCH1* restores BH_4 levels and normalizes eNOS function (64).

Findings from a few *in vivo* studies of GTPCH1 regulation in vascular disease contrast with those of the acute *in vitro* studies described above. In particular, a recent study showed that the common GTPCH1 variant, C + 243T, in the 3′-untranslated region predicted diastolic and systolic BP in individuals with extreme BP (primarily in females) as well as renal NO excretion, but not catecholamine secretion (126). The hyperphenylalaninemia (*hph-1*) mouse model, which is generated by *N*-ethinyl-*N*-nitrosourea mutagenesis of the GTPCH1 locus, also displays reduced levels of BH_4, NO, catecholamines, and serotonin metabolites (63). In this model, deficient BH_4 biosynthesis results in PH, even under normoxic conditions and greatly increases susceptibility to hypoxia-induced PH. Conversely, augmented endothelial BH_4 synthesis through targeted transgenic overexpression of GTPCH1 prevents hypoxia-induced PH. Besides, restoring endothelial BH_4 levels in hph-1 mice by crossing these animals with GTPCH1 transgenic mice prevents PH.

Several recent publications have confirmed that stachydrine, an active component in Chinese medicine, effectively reversed the Hcy-induced endothelial dysfunction and prevented eNOS uncoupling by increasing the expression of GTPCH1 and DHFR (123). Similarly, metformin, one of the most widely used antidiabetic drugs worldwide, is reported to improve the fluctuating glucose-induced endothelial dysfunction in HUVECs. The protective effect of metformin may be mediated through activation of GTPCH1-mediated eNOS recoupling and inhibition of NADPH oxidase *via* an AMPK-dependent pathway (5).

Oxidative Stress, Zinc Release, and GTPCH1

GTPCH1 enzyme activity is regulated by several mechanisms that vary among different cell types and include protein expression (5), post-translational modifications, and association with the regulatory GFRP. Zinc is important for maintaining the structure and function of GTPCH1. The human as well as bacterial enzyme was shown to contain an essential zinc ion that bound the sulfhydryl groups of the Cys-110 (-141) and Cys-181 (-212) and His-113 (-143) (Zinc1Cys2His1 complexation) in each active site of the homodecameric enzymes (Fig. 5) (127). The zinc ion is proposed to generate a hydroxyl nucleophile for the attack of imidazole ring carbon atom eight of the substrate, GTP. The zinc ion represents a selective target for oxidants such as $ONOO^-$ and HOCl. Both HOCl and $ONOO^-$ react fast with the positively charged zinc atom resulting in its release from zinc-containing proteins (Fig. 6). Indeed, the reaction of zinc/thiolate clusters with $ONOO^-$ ($5.2 \times 10^5\ M^{-1}s^{-1}$) is at least

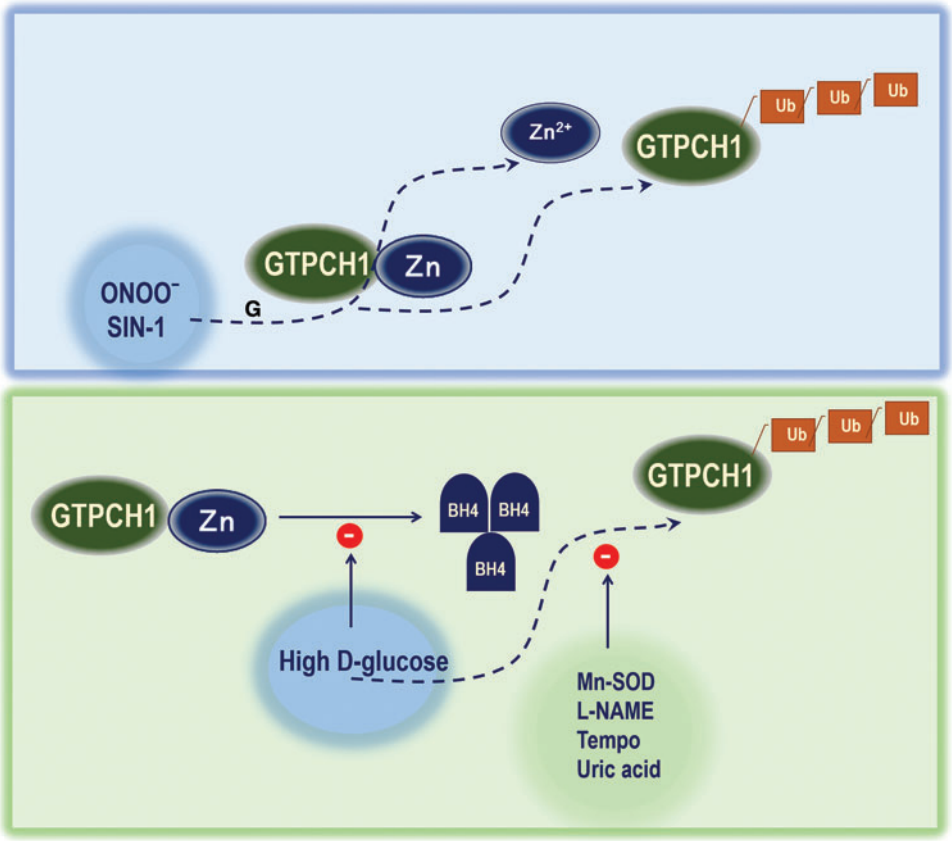

FIG. 6. Zinc releases from GTPCH1 by oxidants and polyubiquitination of GTPCH1. Exposure of $ONOO^-$ or SIN-1, an $NO^{\cdot}$ donor, both target the GTPCH1-Zn complex and release the Zn ion. High-glucose exposure in endothelial cells triggers the GTPCH1 ubiquitination and inhibits BH_4 synthesis. Whereas Mn-SOD overexpression, L-NAME exposure, TEMPO, or uric acid exposure all can inhibit the GTPCH1 ubiquitination triggered by high D-glucose exposure. L-NAME, *N*-nitro-L-arginine methyl ester; SOD, superoxide dismutase. Color images are available online.

1000 times faster than that with cysteine thiols (6×10^2 $M^{-1}s^{-1}$) and 100 times faster than those of BH_4 with $ONOO^-$ (6×10^3 $M^{-1}s^{-1}$) (27).

Our published articles have demonstrated that the zinc structure of GTPCH1 represents a selective target for $ONOO^-$ (127). We found that (a) exposure of recombinant GTPCH1 to $ONOO^-$ or SIN-1, an $ONOO^-$ donor, dose dependently inhibited GTPCH1 activity and releases the zinc ion in parallel with increased GTPCH1 ubiquitination; (b) mutation of the zinc-binding residue cysteine 141 significantly reduced GTPCH1 enzyme activity and reduced its half-life, but increased GTPCH1 ubiquitination, indicating an essential role of the zinc ion in maintaining the catalytic activity and stability of GTPCH1; (c) exposure of endothelial cells to D-glucose, but not an osmotic control, inhibited the enzyme but increased ubiquitination of GTPCH1; (d) pharmacological inhibition of $ONOO^-$ formation with either Tempo, a potent anti-oxidant, or L-NAME, a nonselective NOS inhibitor, or scavenging of $ONOO^-$ with uric acid (a potent $ONOO^-$ scavenger) abolished the HG-induced reduction of GTPCH1 protein levels and activity; (e) adenoviral overexpression of Mn-superoxide dismutase blocked HG-enhanced GTPCH1 degradation; (f) GTPCH1 ubiquitination and degradation markedly increased in parallel with decreased GTPCH1 activity; and (g) supplementation with Tempo prevented the hyperglycemia-induced reduction of GTPCH1 activity, oxidative stress, eNOS uncoupling, and atherosclerosis in diabetic mice *in vivo* (Fig. 6). These data strongly support that oxidation of the zinc-binding structures of GTPCH1 by RNS inactivates the enzyme to trigger its ubiquitination, resulting in eNOS uncoupling and accelerated atherosclerosis in diabetes.

Several recent studies in humans (9, 42, 72, 94, 115) indicate that elevated neutrophils to lymphocyte ratios as a predictor of hypertension and the neutrophil to lymphocyte ratios are correlated with BP variability in hypertensive and normotensive subjects. Elevated white blood cell counts predict an increased incidence of hypertension in the Japanese, especially among females (113). Moreover, neutrophils were the major WBC component contributing to the increased risk for hypertension (113). Consistent with this report, Belen *et al.* report increased neutrophil to lymphocyte ratio in patients with resistant hypertension (9). Taken together, these studies indicate that neutrophil might play a causative role in the development and progression of hypertension. How neutrophils control BP remains unknown.

Activated neutrophil releases hydrogen peroxide and myeloperoxidase, producing hypochlorite, and secondarily, various chloramines. We have provided evidence supporting that HOCl might be another important oxidant that oxidizes and inactivates GTPCH1 in cardiovascular disease. Our unpublished data demonstrate that exposure of recombinant GTPCH1 to pathologically relevant concentrations of HOCl (10–100 μM) for 30 min dose dependently inhibited GTPCH1 activity and reduced its half-life. HOCl increased the ubiquitination of GTPCH1 in endothelial cells. These results indicate the oxidation of the zinc-binding structures of GTPCH1 by HOCl from myeloperoxidase in neutrophils inactivates the enzyme resulting in BH_4 deficiency, with consequent eNOS uncoupling in hypertension.

Potential Pharmacological eNOS/NO Signaling Modulators

NO signaling activators and regulators received widespread attention either as potential therapeutic agents or as preventive agents. Their potential beneficial effects have been largely studied for not just cardiovascular diseases but for many other diseases as well, which include endothelial dysfunction (5), hind limb ischemia (111), cerebral ischemia (6), insulin resistance (82), Parkinson's and Alzheimer's (38), and so on (Table 1). Corticosteroids were shown to exert beneficial effects in the treatment of acute myocardial infarction. Dexamethasone, a corticosteroid, exhibits its cardiovascular protective effects by rapid, nontranscriptional activation of eNOS. Glucocorticoid receptor (GR) is a steroid hormone-activated transcriptional factor. When corticosteroids bind to the GR, it stimulates PI3K/PKA signaling and activates eNOS to trigger its vasorelaxant effect (46). Besides eNOS regulatory elements such as AMPK, PI3K/PKA signaling, Rho/Rho-kinase, the PPAR gamma also is known to regulate the eNOS expression (7). Intriguingly, a huge number of studies showed a key role of statins in inducing the expression of

Table 1. Studies Investigating Endothelial Nitric Oxide Synthase Modulators for Targeting Hypertension

Disease models	*eNOS modulator*	*Effect on NO signaling members*	*Outcome*	*References*
SHR	Rhynchophylline	Rhy activates Src-PI3K/Akt-eNOS signaling pathway	Rhynchophylline ameliorates endothelial dysfunction	(49)
3-MC-mediated murine hypertensive model	3-Methylcholanthrene	3-MC reduced the interaction between eNOS and Akt1. Increased interaction between eNOS and caveolin-1	Simvastatin reduced 3-MC-mediated murine hypertension	(19)
eNOS-deficient mice	Spirulina platensis	SP6, a Spirulina peptide exerts endothelium-dependent vasodilation of *ex vivo* vessels, *via* PI3K/AKT signaling	In an experimental model of arterial hypertension, SP6 exerted an antihypertensive effect	(16)
SHRs	Rosiglitazone	Rosiglitazone increased PPARg expression and activated PI3K/PKB/eNOS signaling	PPARγ agonist could improve endothelial function in the young SHRs	(69)
Double KO of LDLR and leptin	Rosuvastatin	Rosuvastatin restores vascular NO signaling	Increased PPARγ expression by rosuvastatin regulates BP, which might be *via* NO mechanism	(28)
ANTU-induced PAH rodent model	Chrysin	Chrysin induces the eNOS level	Chrysin protects against ANTU-induced PAH *via* increasing eNOS level	(116)
L-NAME-induced hypertensive rats	Nebivolol	Nebivolol increased NO, activity and expression of eNOS, p-eNOS, Akt, and p-Akt	Nebivolol treatment reduced systolic blood pressure and ameliorated aortic remodeling	(118)
Monocrotaline (MCT)-induced PAH	Liraglutide	Liraglutide has therapeutic effects on MCT-induced PAH, through eNOS/sGC/PKG and Rho kinase signaling	Liraglutide prevented and reversed MCT-induced PAH.	(65)
SHRs	Infliximab	Infliximab-enhanced AKT/eNOS phosphorylation reduces I-κB (I$\kappa\beta$) and in the aorta	Infliximab prevents the increase of both systolic pressure and left ventricle hypertrophy in SHRs	(35)
SHRs	Irisin	Irisin increased NO production and eNOS phosphorylation in endothelial cells	Irisin lowers blood pressure through the AMPK-Akt-eNOS-NO signaling pathway	(41)
SHRs	Resveratrol	Resveratrol increases intracellular calcium and activates AMPK, and further increases NO production	Resveratrol increases endothelial NO, improves endothelial function, and lowers BP, which depend on calcium-eNOS activation	(70)

AMPK, AMP-activated protein kinase; BP, blood pressure; eNOS, endothelial nitric oxide synthase; LDLR, low-density lipoprotein receptor; L-NAME, *N*-nitro-L-arginine methyl ester; NO, nitric oxide; PAH, pulmonary arterial hypertension; sGC-PKG, guanylyl cyclase-cGMP-dependent protein kinase; SHR, spontaneously hypertensive rat.

eNOS, which could be implicated in statin-associated cardiovascular disease protection (7). Leptin and low-density lipoprotein receptor deficient double knockout mice develop all the features of the human metabolic syndromes, including insulin resistance and hypertension. When these mice were treated with rosuvastatin for 12 weeks, it normalized BP homeostasis in obese dyslipidemic mice independently of changes in body weight or independent of decreases in plasma cholesterol, whereas rosuvastatin effects were nullified when these animals were treated with inhibition of NOS with L-NAME. This indicates that of these effects, statin effects are mediated by increased activity and expression of eNOS (28).

BH_4 and Its Clinical Perspective

BH_4 acts as a critical regulator of eNOS function and suggests that BH_4 is a rational therapeutic target in vascular disease states, particularly for hypertension. The *hph-1* mutant mice have deficient GTPCH1 activity, resulting in lower levels of BH_4 content in their tissues. Compared with wild-type mice, the lung peroxide content was increased, whereas the eNOS expression was decreased in hph-1 animals. As they grow and turn to adults these mice tend to develop PH (10). These all indicate the importance of GTPCH1 activity and BH_4 level in animal systems to keep the BP homeostasis. BH_4 supplementation in preclinical models showed a significant health improvement. In Ang II-induced hypertensive rat models, BH_4 supplementation in drinking water significantly prevented the Ang II effects, such as impaired vascular responses to acetylcholine, hypertension, and heart weight index values. On the contrary, BH_4 also significantly reduced Ang II-induced iNOS expression and nitrotyrosine and superoxide anion formation in rodents, which indicates that it can reduce the uncoupling effect of NOS (59). However, some clinical trials had been done by several groups, many of the outcomes of these trials were not published or yet to be published. Clinical trial: NCT00435331 (NIH trial identifier number) is one of the very first attempts on PH patients using modified BH_4. It is an analogue of BH_4, a thermo- and photostable drug named 6R-BH_4/sapropterin dihydrochloride, which is used as a drug for phenylketonuric (PKU) patients. The rationale behind this trial was that PKU is also associated with BH_4 deficiency and BH_4 treatment showed significant outcomes for these patients (Clinical Trial No. NCT00964236) (22). Thus, the purpose of this study is to determine whether the sapropterin treatment has any complementary or additive effects on the existing treatment in patients with pulmonary arterial hypertension (PAH). Another objective of the study was also to evaluate the change in biochemical markers of endothelial dysfunction and eNOS activity (94a). Another set of the study was carried out in patients with systemic hypertension (Clinical Trial No. NCT00325962). Similar to the previous study, this study also intended to collect the information on the efficacy of 6R-BH_4 on patients. The baseline eNOS activity and endothelial dysfunction after 8 weeks of treatment in subjects with poorly controlled hypertension were also recorded. However, the 6R-BH_4 supplementation to either systemic hypertension or PAH patients did not show significant improvement, and the complete results of the study have not been published yet (1). However, the above clinical trials did not show promising results, a randomized double-blind, placebo-controlled trial showed a positive outcome on hypercholesterolemic patients (25). In this study, hypercholesterolemic patients were given BH_4 (400 mg twice daily) for 4 weeks. To determine the effect of BH_4 supplement, NO release and $O_2\cdot^-$ production were measured in human aortic endothelial cells. Using venous occlusion plethysmography, the endothelium-dependent and independent vasodilatation was also assessed. This study concluded that BH_4 oral treatment could reverse endothelial dysfunction under hypercholesterolemic settings (25). In another randomized-controlled trial, the patients with type 2 diabetes (T2D) and coronary artery disease were involved to know if improving eNOS protects against ischemia/reperfusion-induced endothelial dysfunction (100). The depletion of physiological levels of L-arginine (eNOS substrate) and BH_4 was observed by many preclinical models, and supplying L-arginine (90) or BH_4 could abrogate the endothelial dysfunction (92). Here, the patients were provided with L-arginine and BH_4. Forearm ischemia was induced in 12 patients with type 2 diabetes and coronary artery disease for 20 min, and 60 min of reperfusion followed by intrabrachial infusion of L-arginine (20 mg/min) and BH_4 (500 μg/min). Remarkably, endothelium-dependent vasodilatation (EDV) was significantly reduced at 15 and 30 min of reperfusion following L-arginine and BH_4 infusion. Remarkably, after L-arginine and BH_4 infusion, EDV was observed to be significantly less reduced at 15 and 30 min of reperfusion compared with the saline infusion (100). Considering the importance of the aforementioned studies, to improve the translational significance, it is essential to continue research in this field to manipulate BH_4 to increase the efficacy for treating hypertension.

Conclusion

In this brief review, we have summarized the evidence supporting that oxidation and ubiquitination of GTPCH1 in hypertension, the rate-limiting BH4 synthesis enzyme, GTPCH1, influences oxidative stress caused by eNOS uncoupling, a process that exacerbates oxidative stress in diverse vascular disease states and that the zinc ion in GTPCH1 is sensitive to oxidants such as HOCl and $ONOO^-$. Understanding oxidant-mediated GTPCH1 inactivation, and ubiquitination by oxidants, might help develop new treatment strategies in hypertension that might help to ameliorate the excess vascular diseases associated with the diseases. Besides, the importance of BH_4 bioavailability on systemic and PH is well understood, however, implying as therapeutics needs in-depth research. Given that eNOS uncoupling is now considered a common mechanism for endothelial dysfunction and hypertension, the gained information might also increase the understandings of other vascular diseases, including atherosclerosis, hypercholesterolemia, and heart failure.

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Abbreviations Used

6-PTS = 6-pyruvoyl-tetrahydropterin synthase
AMPK = AMP-activated protein kinase
BH_4 = tetrahydrobiopterin
CaMK-II = calmodulin-dependent protein kinase II
cGMP = cyclic guanosine monophosphate
DHFR = dihydrofolate reductase
DOCA-salt = deoxycorticosterone acetate-salt
EDV = endothelium-dependent vasodilatation
eNOS = endothelial nitric oxide synthase
eNOS-Tg = eNOS transgenic
FAD = flavin adenine dinucleotide
FMN = flavin mononucleotide
GFRP = GTPCH1 feedback regulatory protein
GPER = G protein-coupled estrogen receptor 1
GR = glucocorticoid receptor
GTPCH1 = guanosine 5′-triphosphate (GTP)-cyclohydrolase 1
HNO_2 = nitrous acid
HOCl = hypochlorous acid
hph-1 = hyperphenylalaninemia
HUVEC = human umbilical vein endothelial cells
iNOS = inducible NOS
LDLR = low-density lipoprotein receptor
L-NAME = *N*-nitro-L-arginine methyl ester
N_2O_3 = dinitrogen trioxide
NADPH = nicotinamide adenine dinucleotide phosphate
NIO = N5-(1-iminoethyl)-L-ornithine
nNOS = neuronal NOS
NO = nitric oxide
NO^+ = nitrosyl
NO_2^+ = nitronium ion
$NO_2\cdot$ = nitrogen dioxide
NO^- = nitroxide
$O_2\cdot^-$ = superoxide anion
$ONOO^-$ = peroxynitrite
PAH = pulmonary arterial hypertension
PH = pulmonary hypertension
PKU = phenylketonuric
RNS = reactive nitrogen species
RONOO = alkyl peroxynitrites
ROS = reactive oxygen species
sGC-PKG = guanylyl cyclase-cGMP-dependent protein kinase
SHR = spontaneously hypertensive rats
SOD = superoxide dismutase
$SSNO^-$ = nitroso persulfide
STAT1 = signal transducer and activator of transcription 1
T2D = type 2 diabetes
Thbs4 = thrombospondin-4
VEGF = vascular endothelial growth factor