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Invited Review Article

Pharmacological targeting of endothelial nitric oxide synthase dysfunction and nitric oxide replacement therapy

Huige Li [a,b,*], Ulrich Förstermann [a], Ning Xia [a], Marin Kuntic [b,c], Thomas Münzel [b,c], Andreas Daiber [b,c,**]

[a] Department of Pharmacology, University Medical Center, Johannes Gutenberg University, Mainz, Germany
[b] German Center for Cardiovascular Research (DZHK), Partner Site Rhine-Main, Mainz, Germany
[c] Center for Cardiology, Cardiology I, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany

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ABSTRACT

The Global Burden of Disease Study identified cardiovascular risk factors as leading causes of global deaths and life-years lost. Endothelial dysfunction is a pathomechanism associated with these risk factors and stressors, and is an early predictor of atherosclerosis. Oxidative stress triggers endothelial dysfunction, a hallmark of cardiovascular diseases. Endothelial dysfunction is largely based on impaired endothelial nitric oxide synthase (eNOS) function and activity or down-stream signalling of nitric oxide. Molecules affecting eNOS functionality, eNOS protein itself as well as components of the eNOS down-stream signalling cascade are attractive therapeutic targets for vascular integrity and homeostasis. Potential strategies for the pharmacological exploitation of these targets are highlighted in the present work. Recent advances and future therapeutic strategies for the treatment of cardiovascular and other diseases should be directed against such targets, including targets so far not considered sufficiently as well as lifestyle changes.

1. Introduction

1.1. Global burden of disease and deaths

The Global Burden of Disease Study shows a shift in the impact of risk factors on global disease burden and life expectancy over the past 20 years, shifting from communicable childhood diseases to non-communicable diseases in the aging population [1,2]. Demographic changes and socio-economic advancements have contributed to this shift. Hypertension, air pollution, ischemic heart disease, smoking, and cerebrovascular disease are leading causes of premature mortality and chronic disease burden. Importantly, more than 2/3 of the global burden of disease are currently caused by non-communicable diseases (NCDs) such as diabetes and atherosclerosis, which is projected to further rise to 77 % in 2030 with a disproportionately high contribution of roughly 50 % by cardiovascular diseases to these NCDs (Fig. 1A and B) [3,4]. These leading health risk factors represent a considerable socioeconomic burden on societies and health care systems. According to projections by the US National Institute of Health, the global over-the-counter drug spending will reach 1.6 trillion US dollar by 2025 with a contribution by circulatory medicines of 14.3 % based on the data from 2020 with a global over-the-counter drug market of 1.3 trillion US dollar (Fig. 1C). Likewise, for global drug prescriptions, circulatory and cardiometabolic drugs rank 3rd with a volume of 146 billion US dollar in 2024 and total costs for drug prescriptions of 1.06 trillion US dollar (Fig. 1D). These numbers make the development of new and cost-efficient circulatory medicines a primary goal for academic and pharmaceutical research. Importantly, all of these health risk factors, classical and environmental ones, ultimately lead to endothelial dysfunction, which represents an early (subclinical) correlate of atherosclerosis and accordingly of future coronary artery diseases and ischemic heart events [5,6] (reviewed in Ref. [7]). Oxidative stress, either by mitochondrial dysfunction or by infiltration of activated immune cells and cellular inflammatory activation, represents a unifying concept for the underlying pathophysiology [8].

* Corresponding author. Department of Pharmacology, University Medical Center, Johannes Gutenberg University, Mainz, Germany.
** Corresponding author. Center for Cardiology, Cardiology I, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany.
E-mail addresses: huigeli@uni-mainz.de (H. Li), daiber@uni-mainz.de (A. Daiber).

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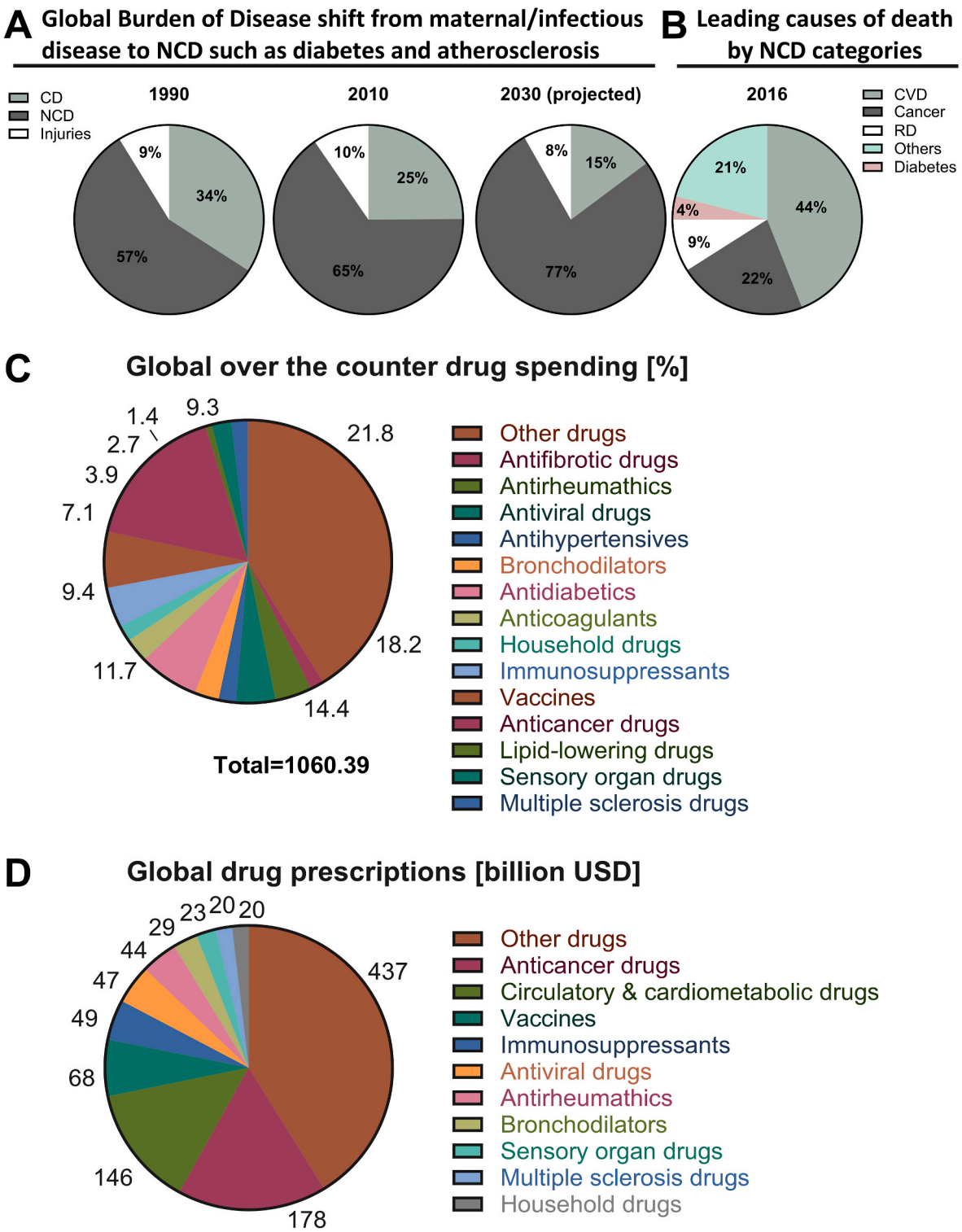

Fig. 1. Global burden of non-communicable disease and medicines costs by different disease segments. (A) The proportional distribution of global deaths attributable to communicable diseases, non-communicable diseases, and injuries for 1990, 2010 and projected for 2030. Graphs generated from previous data [9,10]. (B) Global World Health Organization status report on non-communicable diseases 2016 estimate the leading causes of death by different categories. The category 'Others' comprises neurodegenerative and other neurological diseases, musculoskeletal diseases, congenital anomalies, digestive disorders, endocrine/blood/immune disorders, genitourinary diseases, and other orphan disease categories. CD, communicable disease; NCD, non-communicable disease; CVD, cardiovascular disease; RD, respiratory disease. Graphs generated from previous data [11]. Presented graph was reused from Ref. [12] with permission. © 2020 Elsevier Inc. (C) Global over-the-counter medicine spending by different disease segments in 2020. Data is based on spending for year ending September 2020. Source: IQVIA Institute for Human Data Science report on "Global medicine spending and usage trends: outlook to 2025" (April 2021). (D) Global costs for prescribed drugs in 2024. Circulatory & cardiometabolic drugs comprise antifibrotics, antihypertensives, antidiabetics, anticoagulants and lipid-lowering drugs. Graph was generated from tabular data in Statista Market Insights (June 2024).

1.2. The oxidative stress concept and cardiovascular disease

Oxidative stress represents a pathomechanistic concept that plays a role in various diseases but particularly in cardiovascular diseases [13,14] and results from an imbalance of formation and removal of reactive oxygen and nitrogen species or from insufficient capacity to repair the emerging oxidative damage [15]. The free radical pair nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$), via peroxynitrite or hydrogen peroxide formation, largely determine redox regulation and oxidative stress conditions in the cardiovascular and other organ systems (Fig. 2) [16]. The existence of superoxide dismutases (SODs) in biological systems suggests that superoxide anion radical ($O_2^{\bullet-}$) contributes to pathophysiological processes and that the expression of SODs is mandatory to protect the organism from oxidative damage by superoxide [17], which is also supported by lethality of genetic deficiency of mitochondrial SOD [18]. The only "real" physiological role of superoxide may comprise host defense as patients with chronic granulomatous disease (*Nox* gene mutations) are more susceptible to infections [19]. In contrast, the degradation product of $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), is involved in redox signaling on a broad basis, e.g. by reversible oxidation of protein thiol groups and homeostasis of eustress [20,21]. The "EU-ROS" COST Action position paper provides more examples on H_2O_2-dependent redox signaling [22], suggesting that unspecific systemic inhibition of ROS formation may inhibit ROS's physiological functions, potentially leading to adverse health effects in large-scale clinical trials on antioxidants [23]. It must be emphasized that there are many more reactive oxygen species (ROS) that play a role in cellular damage and biological redox processes, e.g. hypochlorous acid (HOCl), singlet oxygen and organic peroxides, but it is beyond the scope of the present review to discuss all of them.

Oxidative stress is associated with an increased all-cause and cardiovascular mortality as demonstrated by a study in 10,622 men using two oxidative stress serum markers [24]. A meta-analysis of 10,684 individuals found that xanthine oxidase (XO) inhibitors reduced the risk of cardiovascular events at least by trend and highly significant in patients with previous ischemic events [25]. More human evidence on a role of oxidative stress for the development of cardiovascular diseases and risk of major adverse cardiovascular events is provided in Refs. [26,27].

1.3. Nitric oxide as a central regulator of vascular tone and susceptibility to oxidative stress

The endothelium is a single, continuous cell layer on blood vessel inner walls, separating intravascular and interstitial compartments. The endothelial cell layer contains up to $5x10^{13}$ cells, with a mass of 1.5 kg and a surface of 1000 m^2, making it a separate organ. It plays a key role in blood circulation, producing vasoactive, growth, and matrix-modulating substances, and exerting essential autocrine/paracrine functions [28]. The endothelium regulates vessel tone, growth, cell migration, extracellular matrix structure, smooth muscle cell proliferation, and inhibits platelet aggregation and monocyte activation and blood clotting, e.g. by vasoactive substances such as prostacyclin [29] and $^{\bullet}NO$ [30]. The potent vasodilator endothelium-derived relaxing factor (EDRF) was identified as $^{\bullet}NO$ after two decades of research, a joint effort by Nobel Prize recipients Murad, Ignarro, and Furchgott [31–33]. Nitric oxide is a key substance in endothelial function, formed from L-arginine by endothelial nitric oxide synthase (eNOS) and other isoforms. The activity of eNOS is regulated by hormones, cytokines, and mechanical factors, and is controlled by Ca^{2+}/calmodulin, BH_4, and

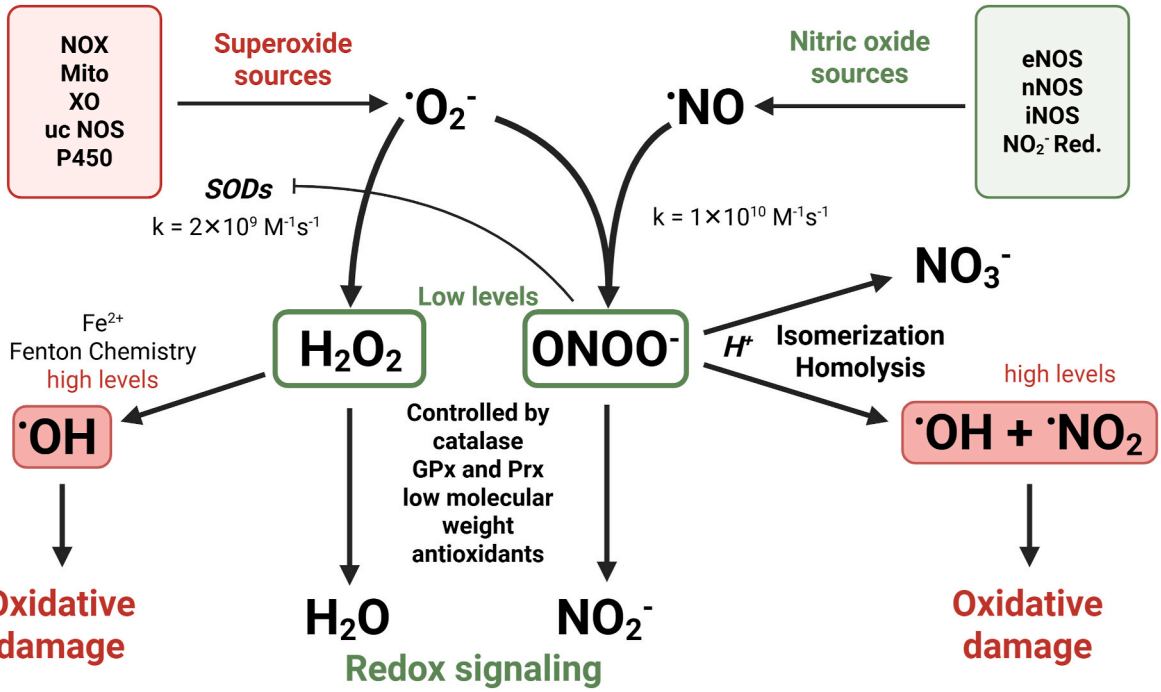

Fig. 2. Vascular oxidative stress and redox signaling are major pathways in the body. Redox signaling is primarily based on H_2O_2, which is formed through the breakdown of $O_2^{\bullet-}$ through self-dismutation or catalyzed by SODs. Biological $O_2^{\bullet-}$ sources include NADPH oxidases, mitochondrial respiratory chain (Mito), xanthine oxidase (XO), uncoupled NOS (uc NOS), and P450 enzyme side reactions. H_2O_2 also modifies enzymatic activities, such as zinc-finger-motifs in transcription factors. Reactions with thiol groups in peroxiredoxin proteins (Prx) or selenol-group in glutathione peroxidases (GPx), catalase but also low molecular weight antioxidants represent major detoxification routes. H_2O_2 accumulation leads to the Fenton reaction and hydroxyl radical formation by iron catalysis, causing severe oxidative damage at protein, lipid, and DNA levels. Biological $^{\bullet}NO$ sources include neuronal, endothelial, or inducible NOS, and nitrite reduction from nutritional sources. The diffusion-controlled reaction of $^{\bullet}NO$ with $O_2^{\bullet-}$ yields $ONOO^-$ anion, which outcompetes the fast breakdown of $O_2^{\bullet-}$ by SOD making $ONOO^-$ formation feasible under physiological and pathophysiological conditions. $ONOO^-$, especially ONOOH, has a 100-1000-fold higher reactivity than H_2O_2, confers similar redox reactions and can be degraded by spontaneous isomerization to nitrate or activated by homolysis to form $HO^{\bullet}$ and nitrogen dioxide radicals ($^{\bullet}NO_2$), similar to the Fenton reaction. Scheme is drawn de novo and modified from Refs. [39,40].

amino acid residue phosphorylation [7]. The key biological activity of •NO is the relaxation of vessels by activation of the guanylyl cyclase in the smooth muscle, formation of cGMP, stimulation of cGMP-dependent protein kinase leading to a decrease of intracellular Ca^{2+} and suppression of myosin light chain kinase. The discovery of free radicals acting as messengers challenged the negative perception and revealed their role in cellular redox signaling and as crucial physiological messenger molecules.

In the 1990s, it was discovered that $O_2^{\bullet-}$ reacts with •NO, forming peroxynitrite ($ONOO^-$) [34,35]. The oxidative potential of $ONOO^-$ is driven by hydroxyl radicals [36], and its nitrating potential is enhanced by carbon monoxide or transition metal centers [37,38]. In the following sections we will present evidence that $O_2^{\bullet-}$ represents a direct antagonist for the potent vasodilatory effects of •NO but also acts as a modulator of •NO-dependent biological activities by shifting them to pro-oxidative ($ONOO^-$) pathways (Fig. 2).

2. Nitric oxide and superoxide reaction to peroxynitrite - superoxide as an antagonist of nitric oxide

$O_2^{\bullet-}$ acts as a direct antagonist of •NO, contributing to endothelial dysfunction by oxidatively degrading it [41]. The generated $ONOO^-$ damages vascular proteins like eNOS [42], soluble guanylyl cyclase (sGC) [43], and prostacyclin synthase (PGIS) [44], known hallmarks of vascular dysfunction [45]. Endothelial (vascular) dysfunction of the micro- and macrovascular system also represents a major health risk of diabetic patients [46]. The interplay and steady-state levels of $O_2^{\bullet-}$, •NO, $ONOO^-$ and their degradation products, along with their control by antioxidant enzymes, determines cellular redox state (Fig. 2) [22]. Low concentrations of ROS can act as messengers in redox signaling, while high concentrations cause oxidative stress and damage.

2.1. Direct scavenging of nitric oxide by superoxide under formation of peroxynitrite

$O_2^{\bullet-}$ reacts with •NO at a rate constant of $1.9 \times 10^{10}\ M^{-1}s^{-1}$, forming $ONOO^-$ [34]. This antagonizing effect on •NO was proven in 1986 by preventing the loss of vasodilatory effects of •NO, formerly known as EDRF, by adding SOD [47]. Gryglewski and coworkers transferred •NO from bradykinin-stimulated cultured endothelial cells (•NO containing perfusate) to an organ bath with endothelium-denuded aortic rings. They observed that extending the distance between the endothelial cell culture and the organ bath caused more inactivation of •NO, which was reversed by adding SOD. The footprints of $ONOO^-$ in vivo can be detected by specific antibodies against 3-nitrotyrosine-positive proteins [48], e.g. in atherosclerotic plaques [49]. Also sulfur oxidations, e.g. in methionine, represent typical post-translational oxidative amino acid modifications by $ONOO^-$. A summary of these findings is shown in Fig. 3. Diminished •NO was shown for angiotensin-II induced arterial hypertension in mice, an animal model known for significant vascular superoxide formation due to exacerbated phagocytic NADPH oxidase activity [50]. The observation that administration of liposome-encapsulated SOD to angiotensin-II infused rats caused a dramatic improvement of impaired endothelium-dependent relaxation supports the concept that vascular superoxide causes oxidative degradation of nitric oxide to peroxynitrite [51,52].

2.2. Impact of peroxynitrite and oxidative stress on eNOS function

A hallmark of most cardiovascular diseases is the imbalance between ROS (e.g., $O_2^{\bullet-}$, H_2O_2, $ONOO^-$ and HOCl) formation and detoxification by low molecular weight antioxidants or ROS degrading enzymes or insufficient repair of oxidative damage [15]. Oxidative stress represents a key mechanism in the development and progression of cardiovascular disease [53] and increased ROS formation leads to uncoupling of eNOS [54]. The "kindling radicals" or "bonfire" hypothesis is based on the concept that the initial formation of $O_2^{\bullet-}$ and subsequent formation of $ONOO^-$ cause secondaryoxidative damage of eNOS, which then in the uncoupled state itself contributes to superoxide production [39]. This ROS-induced ROS formation can also cross-activate mitochondrial sources of oxidative stress [55] or activate xanthine oxidase and NADPH oxidase isoforms [56]. In general, •NO can be antagonized by $O_2^{\bullet-}$, leading to the formation of cytotoxic oxidant $ONOO^-$ [34,47].

eNOS activity is regulated by classical mechanisms like calcium/

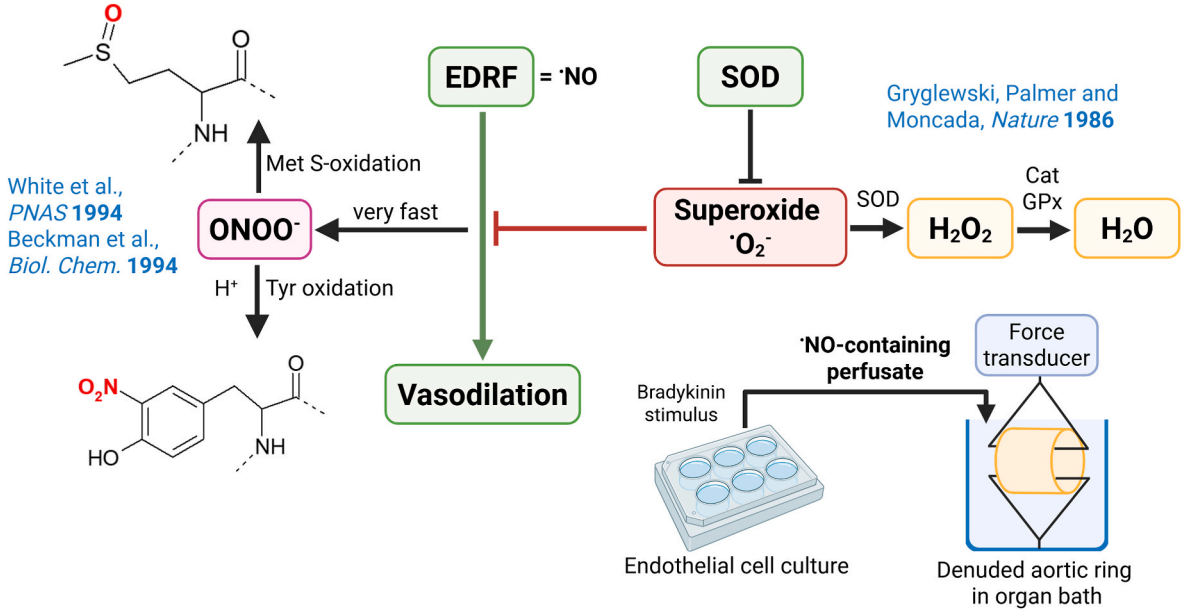

Fig. 3. Scavenging of nitric oxide by superoxide. The simplified model of redox biology in the vascular system identifies $O_2^{\bullet-}$ as an antagonist of the EDRF, now known as •NO. The vasodilatory potency of EDRF is increased by adding SOD to the buffer, supporting the breakdown of EDRF by $O_2^{\bullet-}$. The reaction between •NO and $O_2^{\bullet-}$ forms $ONOO^-$, which can cause widespread oxidative damage to biomolecules, e.g. resulting in tyrosine nitration of methionine oxidation. Without this reaction, $O_2^{\bullet-}$ is dismutated by SODs or spontaneously self-dismutated to form H_2O_2 that is further degraded by catalase (Cat) or glutathione peroxidase (GPx). Scheme is redrawn from Ref. [41] with permission.

calmodulin binding, caveolin interaction, HSP90 association as well as post-translational modifications. While palmitoylation and myristoylation control eNOS subcellular localization, phosphorylation by Akt or adenosine monophosphate-activated protein kinase (AMPK) at Ser1179 stimulates eNOS activity. In addition, redox switches play a major role in eNOS functional state and uncoupling process (for detailed review see Refs. [39,56]). Oxidative depletion of tetrahydrobiopterin (BH_4) [57], oxidative disruption of the dimeric eNOS complex by oxidation of the zinc-sulfur-complex [58], S-glutathionylation of a cysteine in the reductase domain [59] and adverse phosphorylation at Thr495/Tyr657 by PKC/PYK-2 [60,61] as well as ROS-triggered increases in levels of the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA) and diminished ADMA degradation by dimethylarginine dimethylaminohydrolases (DDAHs) [62] under oxidative stress conditions contribute to eNOS uncoupling (Fig. 4). This process is characterized by leakage of electrons from the transport chain

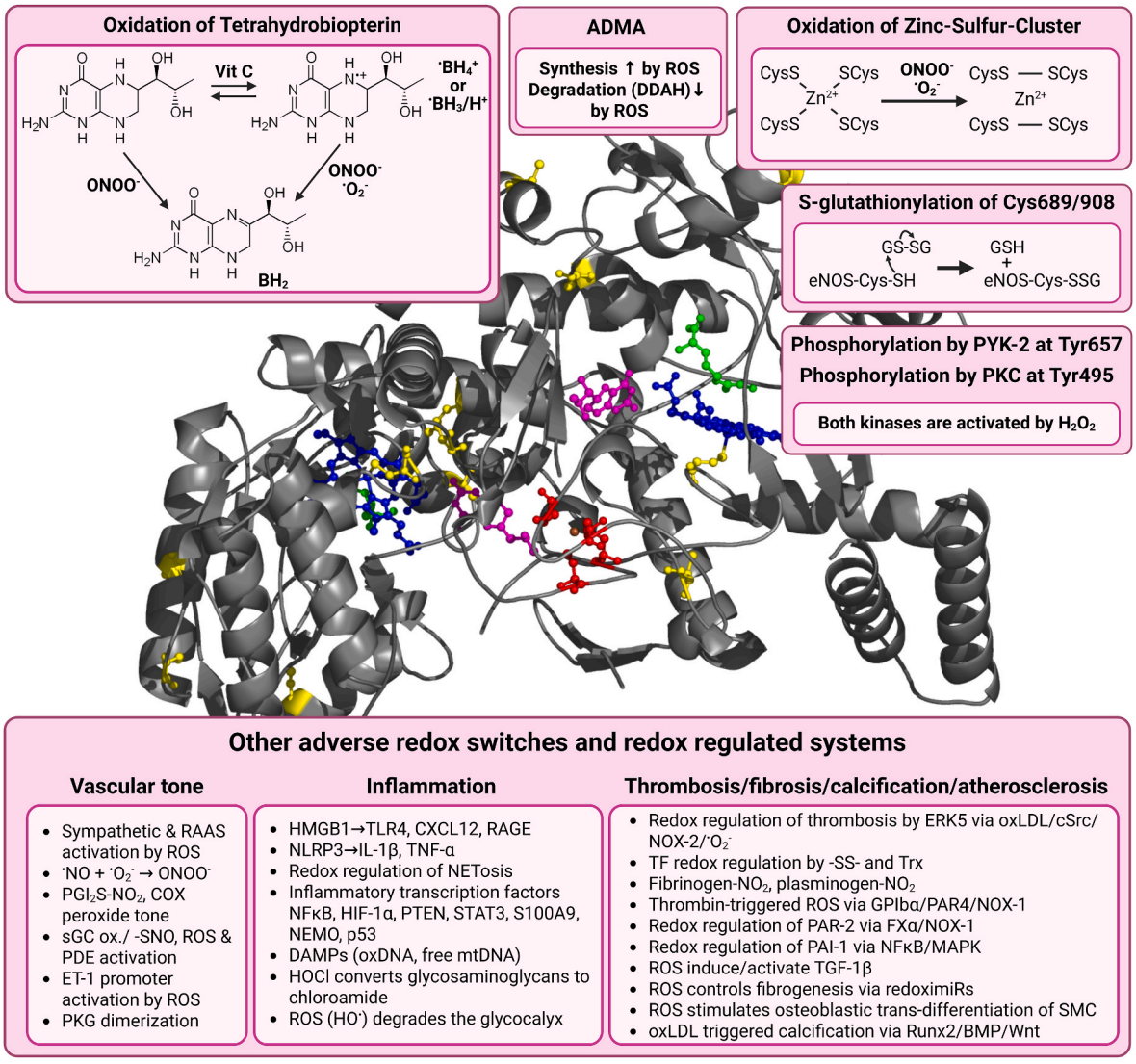

Fig. 4. Mechanisms of eNOS uncoupling. X-ray structure of human eNOS with the iron-porphyrin (blue), the substrate L-arginine (green), the P450-forming axial iron-thiolate ligand from a cysteine residue (yellow), the cofactor tetrahydrobiopterin (BH4) (purple), the zinc-thiolate complex forming cysteines (red, two from each subunit), and the zinc ion (brown). The boxes represent the ''redox switches'' in eNOS, such as S-glutathionylation, protein kinase C (PKC)- and protein tyrosine kinase-2 (PYK-2)–dependent phosphorylation, oxidative BH4 depletion, disruption of the zinc-sulfur cluster, as well as asymmetric dimethylarginine (ADMA) synthesis/degradation, all of which contribute to the regulation of its enzymatic activity. Other downstream redox regulated processes are shown in the box below (adapted from Ref. [65] with permission). GSH, glutathione; GSSG, glutathione disulfide. $ONOO^-$, peroxynitrite; $\cdot O_2^-$, superoxide; DDAH, dimethylaminohydrolase; RAAS, renin-angiotensin-aldosterone system; $PGI_2S\text{-}NO_2$, nitrated prostaglandin I2 synthase; COX, cyclooxygenase; sGC, soluble guanylyl cyclase; -SNO, S-nitros(yl)ation; PDE, phosphodiesterases; ET-1, endothelin 1; PKG, cGMP-dependent protein kinase; HMGB1, high-mobility group box 1; TLR4, toll like receptor 4; CXCL12, stromal cell-derived factor 1; RAGE, receptor for advanced glycation end-products; NLRP3, NLR family pyrin domain containing 3; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; HIF-1, hypoxia-inducible factor 1; PTEN, phosphatase and tensin homolog; STAT3, signal transducer and activator of transcription 3; S100A9, S100 calcium-binding protein A9; NEMO, NF-kappa-B essential modulator; p53, cellular tumor antigen p53; DAMPs, damage associated molecular patterns; HOCl, hypochlorous acid; HO·, hydroxyl radical; ERK5, extracellular signal-regulated kinase 5; oxLDL, oxidized low density lipoprotein; cSrc, proto-oncogene tyrosine-protein kinase Src; NOX-2, NADPH oxidase 2; TF, tissue factor; GPIbα, glycoprotein Ib; PAR-2/4, protease-activated receptor 2/4; NOX-1, NADPH oxidase 1; FXα, activated factor X; MAPK, mitogen-activated protein kinase; PAI-1, plasminogen activator inhibitor-1; TGF-1β, Transforming growth factor beta; SMC, smooth muscle cells; Runx2, runt-related transcription factor 2; BMP, bone morphogenetic protein; Wnt, proto-oncogene Wnt signaling. The crystal structure was rendered from the protein database entry 3NOS (https://doi.org/10.2210/pdb3nos/pdb) using the PyMOL Molecular Graphics System Version 1.2r1 (DeLano Scientific LLC). Redrawn and modified from Ref. [56] with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in the reductase domain (from NADPH over FMN and FAD) and transfer to molecular oxygen yielding superoxide instead of •NO, thereby switching the enzyme from a nitric oxide producer to a superoxide source [42,54]. eNOS dysfunction is correlated with endothelial dysfunction in coronary and peripheral vessels, which can be measured in humans by flow-mediated dilation (FMD) and is an early predictor of cardiovascular events and atherosclerosis [63,64]. Also down-stream of eNOS, the soluble guanylyl cyclase (sGC) can be inactivated via thiol oxidation, S-nitros(yl)ation or ROS-triggered loss of the heme-group [7]. Likewise, cGMP-degrading phosphodiesterases are activated by ROS [42].

2.3. Role of eNOS in perivascular adipose tissue and other vascular cellular compartments for vascular function

Perivascular adipose tissue (PVAT) plays a critical role in regulating vascular homeostasis by producing a large number of bioactive molecules including NO [66–68]. PVAT is a heterogeneous and dynamic tissue composed of adipocytes, endothelial cells, immune cells, stromal progenitors, fibroblasts, and is innervated by sympathetic nerves. The eNOS enzyme is expressed in both PVAT adipocytes and endothelial cells [69,70]. PVAT NO contributes to the anti-contractile effects of PVAT and enhances vasodilation under physiological conditions. In addition, NO derived from PVAT adipocytes also inhibits vascular inflammation, oxidative stress and vascular remodeling by regulating the expression of adipokines and cytokines [68].

Functional eNOS expression in PVAT is essential for vascular health, as shown in our recent study [71]. Selective deletion of adipocyte eNOS in mice results in PVAT inflammation as well as oxidative stress and fibrosis of the vascular wall. Conversely, PVAT NO protects the vasculature from oxidative stress and remodeling, at least partly, by limiting the expression of chemerin, a novel adipokine [72–74]. These findings underscore PVAT-eNOS as a critical mediator of local vascular integrity.

Under conditions of obesity, PVAT undergoes structural and functional changes, associated with disruption of PVAT's normal anti-contractile function, transforming PVAT from a vasoprotective tissue into a contributor to vascular pathology [68]. The "obesity triad"—hypoxia, inflammation, and oxidative stress—collectively impairs eNOS function [68]. While an adaptive or compensatory increase in NO production from mesenteric PVAT was observed during the early phase of high-fat diet (HFD)-induced obesity in mice [75], prolonged HFD feeding resulted in reduced eNOS expression in the mesenteric PVAT of obese rats [76] and in the thoracic aortic PVAT of mice [77].

In addition to reduced eNOS expression, HFD-induced obesity is also associated with abnormal eNOS post-translational modifications. PVAT eNOS phosphorylation at serine 1177 is reduced, because of impaired activity of Akt and AMPK. In contrast, eNOS acetylation at lysine residues 494 and 504 is enhanced in PVAT from obese mice due to a reduced activity of sirtuin-1 (SIRT1) [78,79]. O-GlcNAcylation is another post-translational modification that affects eNOS enzymatic stability, functional activity, and intracellular distribution [80]. Enhanced O-GlcNAcylation of PVAT eNOS has been observed in hyperglycemic human patients, in high sugar diet-fed rats, as well as in offspring of HFD-fed rat dams [81,82]. All these dysregulated post-translational modifications lead to reduced eNOS activity.

In addition, obesity is associated with an induction of arginase expression in PVAT, which depletes L-arginine, the primary substrate for eNOS. The deficiency of L-arginine promotes the development of eNOS uncoupling, producing superoxide at the cost of NO, thereby exacerbating oxidative stress and further reducing NO levels [69]. Notably, the existence of eNOS uncoupling doesn't mean that the eNOS enzyme produces superoxide only. As discussed in our previous review, eNOS uncoupling is not an all-or-none phenomenon [83]. Instead, part of the eNOS enzyme is uncoupled (producing superoxide) whereas some other eNOS molecules remain coupled (producing NO). Under the conditions of diet-induced obesity, the coupled molecules seem to exceed the uncoupled ones, so that the net effect of eNOS is still protective. This may explain why adipocyte-specific deletion of eNOS in obese mice potentiates high-fat diet-induced hypertension despite the existence of eNOS uncoupling. Therefore, feasible strategies to improved PVAT function should aim to improve eNOS functionality rather than to silence eNOS.

PVAT from obese mice exhibits increased macrophage infiltration, upregulation of NADPH oxidase, and induction of arginase expression. Interestingly, these alterations—along with reduced eNOS phosphorylation and enhanced eNOS acetylation—appear to be specific to PVAT and are not observed in the vascular wall itself [78,79]. Consistently, acetylcholine-induced, NO-mediated vasodilation is impaired only in aortic rings with intact PVAT, whereas PVAT-free aortic segments retain normal endothelial function. This highlights the central role of PVAT in mediating obesity-induced vascular dysfunction, while the endothelial layer remains functionally intact under these experimental conditions [78,79].

Several pharmacological strategies aiming to restore PVAT eNOS function have been examined under experimental settings. Ex vivo incubation of PVAT from obese mice with L-arginine and an arginase inhibitor is able to overcome L-arginine deficiency, recouple eNOS and restore NO production [69]. The enhanced eNOS acetylation in obesity can be reversed by SIRT1. Treatment of PVAT from obese mice with NAD^+, the cofactor of SIRT1, results in an improvement of eNOS function [78]. Oral treatment of fructose-fed rats with resveratrol and metformin improves PVAT function by normalizing AMPK phosphorylation and SIRT1 expression [84]. Furthermore, a special Crataegus extract has been shown to restore PVAT function in diet-induced obese mice in vivo by normalizing PVAT eNOS phosphorylation and acetylation [79]. For reasons that remain unclear, the effects are primarily restricted to PVAT [79]. The treatment enhances eNOS phosphorylation in PVAT but does not influence eNOS phosphorylation in epididymal fat or affect body weight [79]. Another potential therapeutic option is the use of glucagon-like peptide-1 (GLP-1) receptor agonists. For example, liraglutide has been shown to enhance eNOS phosphorylation and restore PVAT function in obese mice [85]. Given the emerging role of PVAT in vascular health, therapies aimed at restoring eNOS function in PVAT may represent novel interventions for obesity-associated cardiovascular diseases. Nevertheless, a major challenge remains the development of delivery systems that specifically target PVAT. Although nanoparticle-based strategies conjugated with adipose-homing peptides have been explored for adipocyte-specific targeting, their success has been limited to date [86,87].

To make the story even more complicated, it was shown that eNOS in red blood cells independently of the endothelium contributes to regulation of blood pressure and vascular tone [88]. This conclusion was based on comparing vascular function, hemodynamics, and nitric oxide metabolism in red blood cell-specific eNOS knockout or knock-in mice with the respective endothelial specific genetically modified mice. Red blood cell eNOS protects against ischemia/reperfusion damage in mice [89]. The interplay of red blood cell and endothelial eNOS activity contributes to the complex redox environment of the vasculature [90]. Decreasing the levels of extracellular hemoglobin, a sink of bioavailable •NO, can improve endothelial function in mice and patients with aortic valve stenosis [91]. This observation provides a link to the enigma that •NO formed by red blood cell eNOS should be rapidly scavenged by binding to hemoglobin and free heme. However, according to recent data, NO-ferroheme species can display vasodilatory activity and therefore behaves as a signaling entity in the vasculature, also potentially preserving its vasoactive properties under oxidative stress conditions [92]. This is in line with findings that a critical cysteine residue for S-nitroso species formation in hemoglobin is not required for efficient export of from red blood cells, again supporting a role for nitrosyl-iron species in this process [93].

3. Endothelial dysfunction as an early marker of subclinical atherosclerosis and predictor of future cardiovascular diseases and events

The most important methods to assess endothelial function in humans are FMD (shear stress- and hyperemia-induced vasodilation in the peripheral vasculature) and plethymography (usually based on acetylcholine infusion-dependent vasodilation in the peripheral vasculature), peripheral artery tonometry (also measured in the fingertip) and the classical catheter-assisted acetylcholine test (vasodilation of the coronary vessels by acetylcholine) as well as some less frequently used techniques (reviewed in Refs. [7,45]). Forearm plethysmography and FMD largely reflect endothelial nitric oxide formation since eNOS inhibitors efficiently block the vasodilatory response [94,95]. Endothelial dysfunction is linked to various cardiovascular risk factors, including arterial hypertension [6], hypercholesterolemia [5], diabetes mellitus [96], obesity, and chronic smoking [97]. It is also associated with chronic inflammation markers like C-reactive protein and cardiovascular risk predictors like adiponectin and brain natriuretic peptide. Additionally, traffic noise exposure [98], ambient air pollution [99], and mental stress [100] can also contribute to endothelial dysfunction. Studies have shown that hypercholesterolemia or chronic smoking can cause moderate impairment of endothelial function, while both risk factors combined can cause severe dysfunction [101]. The Gutenberg Health Study found a strong correlation between pro-atrial natriuretic peptide and non-invasive measurement of conduit artery and peripheral arterial performance [102]. A meta-analysis of 15 cohort studies reported a significant correlation of endothelial function measurements using FMD with diagnosed atherosclerosis in patients [103]. However, endothelial function measurements have been found to fail to predict cardiovascular events in mostly healthy subjects and individuals with intermediate cardiovascular risk, and measurements of intima-media thickness and stiffness index may be recommended for more accurate endothelial and vascular function determination (reviewed in Ref. [7]).

4. Therapeutic targeting of endothelial dysfunction and downstream nitric oxide signaling

4.1. Targeting of endothelial nitric oxide synthase dysfunction

Sepiapterin has been shown to improve endothelial function in atherosclerotic mice in vivo [104] or in hypertensive mice ex vivo [105], suggesting it could also prevent eNOS uncoupling in vascular tissues. The concept of eNOS "recoupling" by administering BH4 or its precursors, sepiapterin and folate, was supported by small-size cohort human studies. Clinical evidence for the role of oxidative BH4 loss is based on improvements in endothelial function in chronic smokers by BH4, but not by tetrahydroneopterin (NH4), which shares the same antioxidant properties with BH4 but is not a cofactor for eNOS [95]. BH4 administration also improved endothelial function in diabetic patients [106]. Supplementation with the BH4 analogue folic acid improved endothelial function in human subjects [107,108]. Co-therapy with propranolol and the BH4 precursor 5-methyltetrahydrofolate decreased the hepatic venous pressure gradient in patients with liver cirrhosis more efficiently than propranolol alone [109]. More examples of small human studies with positive outcome of BH4-targeting therapy were summarized in Refs. [7,110]. However, despite promising preclinical data and findings of small interventional human studies on a protective role of BH4 and precursors, other randomized multicentre studies using sapropterin (a synthetic BH4 analog) therapy in patients with stroke and cirrhosis failed to confirm these benefits [111,112]. In another clinical study, patients with coronary artery disease undergoing bypass surgery received oral BH4 administration but showed no improvement of vascular function as measured by different methods [113]. Based on the plasma levels of BH4 and its oxidation product dihydrobiopterin (BH2), the authors concluded that oxidation rate of orally administrated BH4 is too fast to cause improvement of vascular function in the coronary arteries. Therefore, the clinical data are so far inconclusive and warrant larger clinical trials on the protective effects of BH_4 analogs in the vasculature, the heart and cerebral vessels.

A clinical study from 2025 on patients with peripheral artery disease reported that a combined oral therapy with L-citrulline and BH4 improved the absolute claudication distance as a surrogate measure of improved vascular function [114]. These impressive results may be due to the combined effects of L-citrulline, e.g. by providing higher L-arginine substrate levels for eNOS and by preventing asymmetric dimethylarginine (ADMA) accumulation and inhibition of eNOS, together with the “recoupling” effects of BH4. The authors showed an inverse correlation of absolute claudication distance with plasma levels of the oxidative stress marker 3-nitrotyrosine, the ROS producing vascular enzyme NOX-5 and ADMA. Accordingly, it may be important to combine BH4 therapy either with potent antioxidants to prevent its fast oxidation in vivo or with another drug supporting proper eNOS function by increasing substrate availability and/or suppressing ADMA synthesis, accelerating ADMA degradation or promoting its removal from endothelial cells via cationic amino acid transporters (as discussed for L-arginine in detail in section 4.4).

Also, despite promising preclinical data from eNOS enhancers like AVE9488 and AVE3085 [115,116], no new clinical therapy has been developed yet. The most beneficial results of an eNOS-enhancing therapy can be obtained when the drugs not only up-regulated eNOS but also the BH4-synthesizing enzyme GTP-cyclohydrolase-1 (GCH-1) [117]. This is because eNOS enhancement is only successful when BH4 levels are also increased, otherwise, it produces superoxide instead of $^{\bullet}NO$ [118]. Indeed, AVE9488 increased BH4 synthesis in a mouse model of atherosclerosis [119]. This has not been demonstrated for AVE3085 so far, although it is plausible given the structural similarity between the two compounds [120]. Therapeutic approaches to target eNOS dysfunction are summarized in Fig. 5 and Table 1.

4.2. Targeting of endothelial nitric oxide synthase phosphorylation, acetylation and S-glutathionylation

Phosphorylation at serine 1177 of human eNOS is a crucial regulatory mechanism that stimulates eNOS enzymatic activity and promotes NO production [80,121], with Akt being the central kinase mediating this activation [122]. A decline in phosphorylation at this site is consistently observed under pathological states associated with oxidative stress such as type 2 diabetes mellitus [123,124] and ischemia–reperfusion injury [125], contributing to endothelial dysfunction and impaired vascular reactivity.

Among established cardiovascular drugs, statins exert several pleiotropic vascular-protective effects that extend beyond lipid lowering. Notably, statins enhance endothelial NO production by upregulating eNOS expression and preventing eNOS uncoupling. Moreover, statins stimulate eNOS serine 1177 phosphorylation, a process largely mediated via the PI3K/Akt and the AMPK signaling pathways [126–128]. These effects help to restore endothelial function, inhibit leukocyte adhesion, and reduce vascular inflammation—hallmarks of early atherogenesis.

Resveratrol, a polyphenolic compound found in red wine and grapes, increases eNOS expression, prevents eNOS uncoupling, and also promotes eNOS activity through multiple, dose-dependent mechanisms [129,130]. At nanomolar concentrations, resveratrol activates the MAP kinase Erk1/2 pathway, whereas micromolar concentrations stimulate AMPK, both culminating in increased eNOS serine 1177 phosphorylation [131–134]. In addition, resveratrol is an activator of SIRT1. SIRT1 targets lysine residues 494 and 504 of human eNOS in the calmodulin-binding domain. Deacetylation of these residues by SIRT1 further enhances eNOS enzymatic activity [135]. This regulatory pathway is particularly relevant under oxidative stress conditions, where cigarette smoke extract or H_2O_2 reduce SIRT1 levels and increase

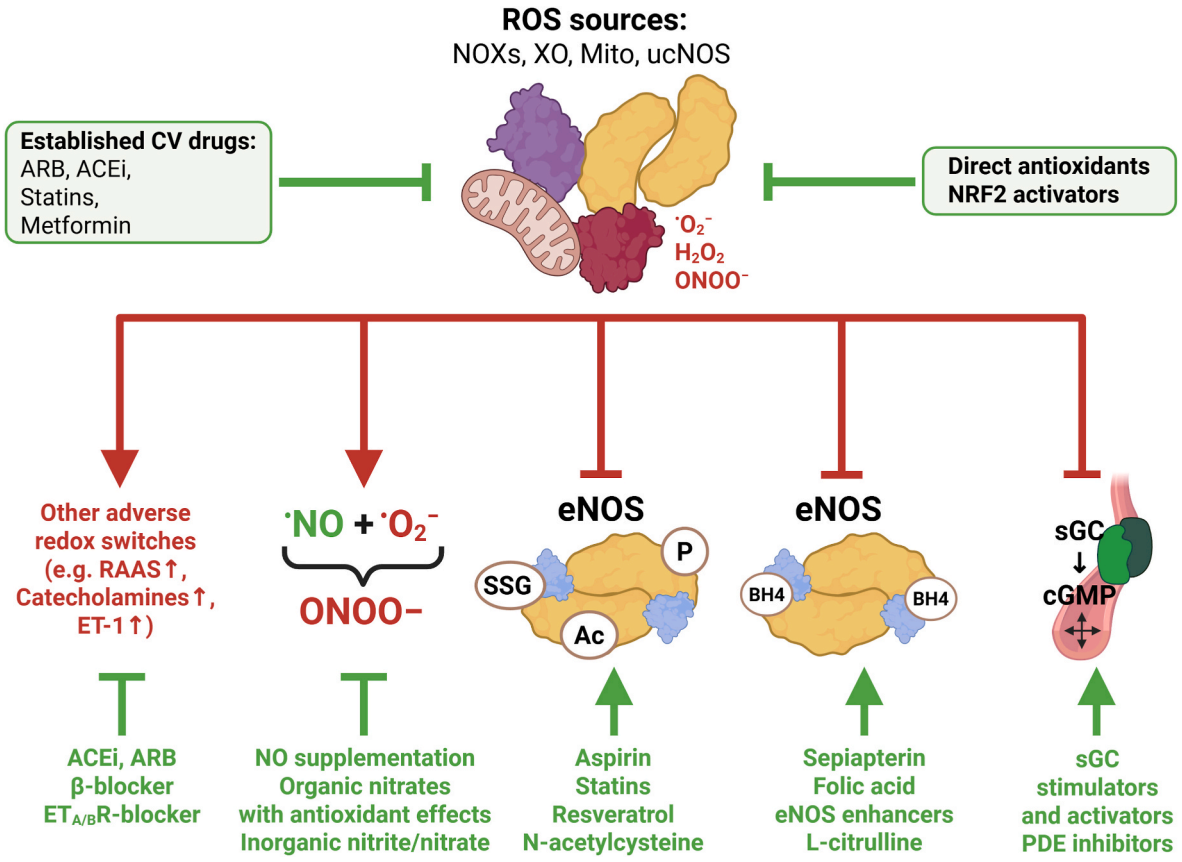

Fig. 5. Overview on therapeutic targets and respective drugs in the eNOS/NO/sGC/cGMP signalling cascade. Targets are the reactive oxygen species (ROS) sources and molecules that are produced, the oxidative break-down or diminished synthesis of nitric oxide (•NO) and impaired cGMP synthesis and bioavailability. Also other redox regulated pathways such as the renin-angiotensin-aldosterone system (RAAS), vasoconstrictor stress hormones (e.g. adrenaline and noradrenaline) and endothelin-1 (ET-1) signaling. Abbreviations: CV, cardiovascular; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin type 1 receptor blocker; ETA/B-receptor blocker; NOXs, NADPH oxidases: XO, xanthine oxidase; Mito, mitochondria; ucNOS, uncoupled nitric oxide synthase; NRF2, nuclear factor erythroid 2-related factor 2; eNOS, endothelial nitric oxide synthase; P, phosphorylation of eNOS; Ac, acetylation of eNOS; SSG, S-glutathionylation of eNOS; BH4, tetrahydrobiopterin; sGC, soluble guanylyl cyclase; PDE, phosphodiesterase.

Table 1
Approaches targeting the NO/cGMP pathway.

	eNOS expression	eNOS function	eNOS phosphorylation	eNOS acetylation	NO replacement	sGC	PDE inhibition
eNOS enhancers	+	(+)					
Sepiapterin		+					
Folate		+					
L-citrulline		+					
Statins	+	+	+				
Resveratrol	+	+	+	+			
Aspirin		(+)	+	+			
iNO					+		
Sodium nitroprusside					+		
Organic nitrates					+		
Inorganic nitrite and nitrate					+		
sGC activators						+	
sGC stimulators						+	
PDE inhibitors							+
Nebivolol		+					
Hydralazine		+					

+, action that enhances production of NO and/or cGMP, or potentiates the effects of NO and/or cGMP; (+), plausible but not experimentally confirmed; iNO, inhaled NO.

eNOS acetylation. Resveratrol pretreatment prevents these maladaptive changes and normalizes eNOS acetylation, emphasizing its therapeutic potential in restoring endothelial homeostasis under oxidative stress [136].

Phosphorylation of eNOS at tyrosine 657 and threonine 495 by protein tyrosine kinase-2 (PYK-2) and protein kinase C (PKC) inhibits the NO-producing activity of the enzyme [60,137]. The adverse (inhibitory) phosphorylation of eNOS has been observed under oxidative stress conditions, e.g. in hypertensive mice [61], nitrate-tolerant rats [138] and mice at old age [139]. These observations can be explained by the fact that both kinases are highly redox-regulated via hydrogen peroxide or peroxynitrite-mediated activation [61,140].

Accordingly, the adverse phosphorylation of eNOS may be prevented by removal of reactive oxygen species, e.g. by high dose N-acetylcysteine, or by PKC and PYK-2 inhibitors, although these approaches have so far not been explored in clinical therapy.

Acetylsalicylic acid (aspirin), traditionally used for its COX-1-mediated antithrombotic effects, also demonstrates pleiotropic endothelial benefits via non-COX pathways [141,142]. Aspirin directly acetylates lysine 607 of human eNOS (lysine 609 in bovine eNOS) within the calmodulin autoinhibitory domain, promoting calmodulin binding and enhancing eNOS activity [143]. This mechanism represents a distinct pharmacodynamic action independent of cyclooxygenase inhibition. Notably, histone deacetylase 3 (HDAC3) can reverse this eNOS acetylation, while SIRT1 does not, indicating that different classes of deacetylases target specific eNOS regulatory lysines [143].

Moreover, aspirin influences eNOS phosphorylation indirectly through redox modulation. In human umbilical vein endothelial cells, the pro-inflammatory adipokine resistin induces NADPH oxidase-mediated ROS production, reducing Akt activity and thereby decreasing eNOS serine 1177 phosphorylation. Aspirin pretreatment counteracts these effects by lowering ROS levels, restoring Akt phosphorylation, and preserving eNOS serine 1177 phosphorylation [144]. By inhibiting endothelial ROS production, aspirin may potentially prevent eNOS uncoupling; however, this specific mechanism has not yet been directly investigated.

Together, these findings highlight the critical role of eNOS modulation as a shared mechanism underpinning the pleiotropic cardiovascular benefits of statins, resveratrol, and aspirin. In the case of aspirin, its ability to enhance eNOS activity via both acetylation and redox-sensitive phosphorylation pathways may contribute significantly to its vascular protective profile, particularly in endothelial dysfunction and atherosclerosis, beyond its well-established antithrombotic action. Future pharmacological strategies could leverage these dual regulatory axes—post-translational phosphorylation and acetylation—to fine-tune eNOS activity in a context-dependent manner.

S-glutathionylation of eNOS at a cysteine in the reductase domain represents a direct redox post-translational modification, which leads to uncoupling of the enzyme [59]. Uncoupled S-glutathionylated eNOS in association with impaired endothelial function was found in hypertensive mice, where it was prevented by genetic deletion of NADPH oxidase as a ROS source [50], in noise-exposed mice [145], mice at old age [139] and diabetic rats [146] (more disease conditions reviewed in Ref. [110]). The •NO-producing activity of uncoupled S-glutathionylated eNOS can be recovered by glutaredoxin-1 [147], suggesting that this pathway may be druggable. Indeed, eNOS S-glutathionylation in mice with cardiac pressure-overload was prevented by N-acetylcysteine administration [148]. However, these approaches need further exploration in clinical therapy.

4.3. Targeting of impaired nitric oxide synthase down-stream signaling

The eNOS/NO/sGC/cGMP axis is a key target for pharmacological therapy in various cardiovascular diseases due to its significant redox-regulation [7]. Organic nitrates are used as •NO replacement therapy for stable angina, congestive heart failure, and acute coronary syndromes, despite limitations due to nitrate tolerance and endothelial dysfunction caused by oxidative stress [149]. Some organic nitrates may also be used for pulmonary arterial hypertension and preeclampsia. Pentaerithrityl tetranitrate (PETN) is the only organic nitrate without side effects like oxidative stress, nitrate tolerance, and endothelial dysfunction [149,150]. Its beneficial effects are due to the induction of heme oxygenase-1 [151,152] and numerous other protective genes in a nuclear factor erythroid 2-related factor 2 (NRF2)-dependent manner [153]. Some niche cardiovascular complications show proven benefit from scavenging of ROS to improve endothelial and cardiovascular function, e.g. by N-acetylcysteine and other low molecular weight antioxidants [65], the antioxidant molecules induced by NRF2 activation such as bilirubin [154] or antioxidant enzymes such as SOD or glutathione peroxidase isoforms that are under transcriptional control of NRF2 [153]. •NO donors like sodium nitroprusside are also used for acute hypertensive crisis in emergency rooms. Also inhaled •NO (iNO) is a real success story in the clinics, especially for improved prognosis of premature infants [155] and reducing the time of mechanical ventilation and stay at the intensive care unit for cardiopulmonary bypass patients [156]. These therapeutic agents have shown success in treating various cardiovascular diseases. Reasons for the failure of •NO donors for chronic therapy have been previously discussed in detail [7] and briefly in the preceding sections.

The use of dietary inorganic nitrite and nitrate as a source of endogenous nitric oxide formation is so far not fully explored in humans [157,158], although there is growing evidence that inorganic nitrite and nitrate from beet root or spinach confers highly beneficial effects on cardiovascular function and blood pressure [159–161], even in patients with severe heart failure [162]. Accordingly, inorganic nitrite and nitrate may represent a cheap and affordable NO-based therapy [163,164]. The beneficial cardiovascular effects of nitrite and nitrate supplementation were also confirmed by a meta-analysis reporting blood pressure lowering effects, improvement of endothelial function, reduction of arterial stiffness and inhibition of thrombotic processes [161]. Meta-analysis data of animal studies also clearly points towards a reduction of ischemic damage upon myocardial infarction by nitrite and nitrate administration [165]. Mechanistically, nitrite and nitrate seem to not simply act as •NO donors but also display antioxidant effects such as the suppression of NADPH oxidase activity in macrophages [166,167]. Moreover, nitrite and nitrate can mobilize angiogenic cells, prevent leukocyte recruitment and interfere with inflammatory processes [168,169]. Importantly, there is evidence that nitrite is more efficiently bioactivated to a vasodilator in vessels of diseased animals as shown for spontaneously hypertensive rats [170] and aortic ring segments pretreated with various oxidants [171]. A potential explanation for this observation may be a higher abundance of xanthine oxidase, a bioactivating enzyme of inorganic nitrite [172,173], due to more pronounced oxidative conversion of xanthine dehydrogenase to the oxidase isoform [174,175] in vascular beds with higher oxidative stress [171].

Other mainstay drugs targeting the NO/cGMP signaling pathway include phosphodiesterase inhibitors, which prevent the enzymatic degradation of cGMP, and activators and stimulators of the sGC, which are repair drugs of a damaged sGC enzyme. Phosphodiesterase inhibitors are used in various indications, including erectile dysfunction, pulmonary hypertension, and cardiac diseases, and are reported to prevent endothelial dysfunction, oxidative stress, and inflammation [176]. Activators and stimulators of the sGC are primarily intended for treating pulmonary hypertension, heart failure, but also show benefits in arterial hypertension, renal fibrosis, liver cirrhosis, erectile dysfunction, atherosclerosis, restenosis, thrombosis, and inflammation [177].

Among 5050 patients with severe heart failure, cardiovascular mortality or hospitalizations were significantly lower in the vericiguat (a sGC stimulator) treatment group when compared to those receiving placebo [178]. However, effects reported by meta-analysis were less pronounced. Soluble guanylate cyclase modulation in patients with heart failure showed no significant difference in the risk of all-cause mortality (also reflected by neutral effects on NT-proBNP levels) compared to placebo [179]. Only life quality was clearly improved by the sGC-modulating drugs. Another meta-analysis showed reduced heart failure hospitalization but no significant reduction of cardiovascular or all-cause mortality by sGC-modulating drugs in patients with reduced ejection fraction (HFrEF) but no benefits at all in those with preserved ejection fraction (HFpEF) [180]. A meta-analysis on the effects of riociguat (also a sGC stimulator) in the treatment of chronic thromboembolic pulmonary arterial hypertension found significantly improved hemodynamic parameters, exercise capacity and quality of life [181]. Therapeutic approaches to target NO/sGC/cGMP dysfunction are summarized in Fig. 5.

Rather experimental pharmacological approaches down-stream of eNOS comprise S-nitros(yl)ation of sGC and oxidative dimerization of protein kinase G (cGMP-dependent kinase). sGC was found S-nitros(yl) ated in the setting of nitroglycerin-induced nitrate tolerance [182] and this post-translational redox modification was associated with impaired enzymatic activity [183]. It seems that even inactivated S-nitros(yl)ated sGC can be rescued by treatment of nitrate tolerant rats with an sGC activator [184]. Likewise, protein kinase G was reported to display higher enzymatic activity associated with enhanced vasodilation, when critical cysteine residues form an intermolecular disulfide bridge forming a protein dimer, e.g. in the presence of physiological levels of hydrogen peroxide [185]. The proposed concept was further confirmed by lack of ROS-induced relaxation in protein kinase G mutants without these essential cysteine residues (Cys42Ser) [186]. This mechanism provides an explanation at the molecular level for cGMP-independent vasodilation by oxidants, also suggesting hydrogen peroxide to be the endothelium-derived hyperpolarizing factor (EDHF). Alternatively, electrophiles such as 8-nitroguanosine 3′,5′-cyclic monophosphate can cause activation of protein kinase G via site-specific protein S-guanylation [187]. However, these approaches have so far not been explored in clinical therapy.

4.4. Other drugs to improve eNOS function

L-Arginine: As the substrate for eNOS, L-arginine enhances NO production in cultured endothelial cells [188]. L-arginine is a substrate for both NO synthases and arginase [189]. Under pathological conditions, increased activity of arginase “steals” L-arginine from eNOS [190]. The resulting L-arginine deficiency reduces NO production, and promotes eNOS uncoupling [54]. In animal models of cardiometabolic diseases, L-arginine supplementation has been shown to increase endothelial NO production and improve vascular function [189,191, 192]. Clinical trials have also shown that L-arginine supplementation improves endothelial function in patients with hypertension [193] and ischemic heart disease [194]. It is likely that patients with higher ADMA levels or with lower L-arginine-to-ADMA ratio, may benefit more from L-arginine supplementation [189]. Finally, L-arginine may serve as a potential therapy for impaired y^+L amino acid transporters, such as in genetic deficiencies, where the cationic amino acid transporter (CAT-1) compensates by using cationic amino acids like L-arginine for ADMA export from the cytosol of endothelial cells [195]. L-arginine may also modulate ADMA uptake in the brain [196].

However, some trials fail to show beneficial effects [189,197]. Notably, even harmful effects have been reported if L-arginine is supplemented over a longer period of time. In ApoE/iNOS double knockout mice, L-arginine supplementation for 24 weeks aggravated atherosclerosis [198]. In patients after myocardial infarction, L-arginine supplementation for 6 months did not improve vascular stiffness measurements and was associated with higher postinfarction mortality [199]. In patients with peripheral arterial disease, long-term administration of L-arginine for 6 months did not increase NO synthesis or improve vascular reactivity. Moreover, L-arginine was less effective than placebo in improving functional capacity in treadmill exercise [200]. To study the molecular mechanisms underlying the detrimental effects of long-term L-arginine treatment, human umbilical vein endothelial cells were incubated with L-arginine for 30 min or 7 days. While acute L-arginine administration enhanced endothelial NO production, chronic treatment induced endothelial senescence and eNOS uncoupling, which was associated with upregulation of arginase-II [201]. Similarly, treatment of aged mice (18–24 months) with L-arginine for 16 weeks increased vascular ROS production, an effect that was prevented by arginase II deletion [202]. Moreover, L-arginine supplementation further enhanced age-associated albuminuria and mortality [202]. Therefore, current evidence does not support the recommendation of long-term dietary L-arginine supplementation, particularly in the elderly population.

L-Citrulline: L-citrulline is a non-essential amino acid that is converted into L-arginine via the urea cycle [203]. In young spontaneously hypertensive rats, L-citrulline prevents hypertension development by increasing L-arginine-to-ADMA ratio and NO production [204]. In human patients with hypertension, blood pressure-reducing effects and protection against artery stiffness by L-citrulline have been shown in several studies [197,205]. We have shown recently that L-citrulline ameliorates preeclampsia phenotypes in rats [206], which is consistent with results from another study using a mouse model of preeclampsia [207]. In human patients with hypertension, blood pressure-reducing effects and protection against artery stiffness by L-citrulline have been shown in several studies [205]. However, a recent clinical trial involving 115 patients with preeclampsia reported no beneficial effects of L-citrulline supplementation [208]. It is important to note that in the animal studies, L-citrulline was administered either before pregnancy [206] or from the onset of pregnancy [207] as a preventive measure. In contrast, L-citrulline supplementation was initiated only after the diagnosis of preeclampsia and patient inclusion in the clinical trial, which occurred after 24 weeks of gestation [208]. This difference in timing and duration of L-citrulline supplementation may explain the discrepancy between the encouraging results observed in animal models and the lack of efficacy in the human trial.

Unlike L-arginine, which is rapidly metabolized by arginase in the gastrointestinal tract and liver, L-citrulline has a higher bioavailability and serves as a more efficient precursor for increasing systemic L-arginine levels [209]. Animal studies have demonstrated that L-citrulline supplementation increases plasma L-arginine levels more effectively than direct L-arginine administration [210]. In addition, L-citrulline is more specific for increasing NO production. L-Arginine has multiple metabolic roles beyond being a precursor of NO, which may explain its mixed (including adverse) effects [211]. In contrast, L-citrulline is a precursor for L-arginine in the citrulline–NO cycle without perturbing other metabolic functions, highlighting L-citrulline’s potential advantage over L-arginine [212]. To date, no serious adverse effects have been reported in association with L-citrulline supplementation [197]. Nevertheless, large-scale, long-term clinical trials are required to validate its role in the prevention and treatment of cardiovascular disease, as well as to determine optimal dosing and duration of use.

4.5. Targeting the crosstalk of NO/cGMP with cAMP signaling cascade

There is increasing evidence demonstrating extensive crosstalk between NO/cGMP signaling and cAMP pathways, enabling fine-tuned regulation of endothelial function, vascular smooth muscle relaxation, and cardiac contractility [213]. Specific PDEs represent the major points of interaction between cGMP and cAMP signaling pathways [214].

Stimulation of $G_{\alpha s}$ protein-coupled receptors leads to the activation of adenylyl cyclase and the production of cAMP. Cardiomyocytes express 2 isoforms of cAMP-dependent protein kinase, PKA-I and PKA-II, with different subcellular localization and downstream targets. PKA-I is mainly found in the cytosol, associated with PDE3 and involved in regulation of cell survival and metabolism. In contrast, PKA-II localizes to the signaling microdomain close to the cell membrane, associated with PDE2 and is essential for contractility. Whereas β-adrenoceptor stimulation generates a cAMP pool selectively activating PKA-II, prostaglandin receptor stimulation produces a different cAMP pool activating PKA-I [215]. Interestingly, the effect of cGMP also varies depending on its subcellular localization and source. NO derived from exogenous NO donors activates sGC. The resulting cGMP reaches both the PKA-I and PKA-II compartments. In the PKA-I compartment, cGMP inhibits PDE3 resulting in increased cAMP levels. In contrast, cGMP activates PDE2 the PKA-II compartment leading to a reduction of cAMP concentration. The opposing effects are attributable to the local activity of cGMP-regulated PDEs, with PDE2 being activated and PDE3 inhibited by cGMP. Importantly, natriuretic peptides ANP and BNP activate pGC and the resulting cGMP pool is restricted to the PKA-II compartment. By

activating PDE2 in the PKA-II compartment, cGMP generated by natriuretic peptides reduces cAMP levels and limits cardiomyocyte contractility [216].

Although an acute increase in cAMP level improves cardiac inotropy, it is known that chronic cAMP increase in the heart is associated with higher mortality in heart failure patients [217]. Inhibition of PDE2, PDE3 and PDE4 all increases cAMP levels in cardiomyocytes. Strikingly, inhibition of PDE3 and PDE4 induces hypertrophy whereas inhibition of PDE2 has antihypertrophic effects [218]. Further evidence suggests that the protective effects of PDE2 inhibition is mediated by PKA activation in the PKA-II compartment [218]. This makes PDE2 an interesting target for heart failure therapy. Indeed, PDE2 expression is upregulated both in experimental models and in human patients with heart failure [214], and inhibition of PDE2 has been shown to reduce hypertrophy and cardiac fibrosis in animal models [218,219]. PDE2 hydrolyzes both cAMP and cGMP, and the protective effects of PDE2 inhibition have also been attributed to cGMP signaling [219]. Nevertheless, there are still a number of controversies regarding the effectiveness and mechanisms of PDE2 inhibition in treating heart failure, and PDE2 inhibitors have not yet been tested for cardiovascular disease in humans [214].

In addition to PDE enzymes, crosstalk between the cAMP and cGMP pathways can also occur at other levels. For instance, eNOS is a substrate of PKA, meaning that cAMP production in endothelial cells can lead to cGMP generation through the cascade cAMP – PKA – eNOS – NO – sGC – cGMP [220]. Conversely, NO has been shown to inhibits adenylyl cyclase activity via selective S-nitrosylation [221,222]. Furthermore, cAMP/cGMP crosstalk is also evident in immune cells [223]. Given the important contribution of inflammation to the pathogenesis of cardiovascular disease, targeting cAMP/cGMP crosstalk may potentially leads to the development of novel therapies.

4.6. Established cardiovascular drugs: statins, ACE-inhibitors and AT1-receptor blockers

Chronic activation of the renin-angiotensin-aldosterone system (RAAS) is linked to endothelial dysfunction, atherosclerosis, and late cardiovascular complications. Diabetic patients show beneficial responses to various therapies, including mineralocorticoid receptor blockade [224], angiotensin-converting enzyme (ACE) inhibitor [225] and angiotensin receptor type 1 (AT_1R) blockade [226]. Statin therapy also has been shown to improve cardiovascular complications, on-top of AT_1R blockade [227]. These drugs lower oxidative stress levels and inhibit inflammation by reducing lipids and vasoconstrictors (reviewed in Refs. [27,228–230]). They also improve NO/cGMP signaling by modulating redox switches in this pathway (Fig. 5). Inhibition of the vasoconstrictor receptors of angiotensin-II and endothelin-1 also prevents the formation of diacylglycerol via G-protein-coupled phospholipase activation, which inhibits the activation of protein kinase C by diacylglycerol and thereby phosphorylation and activation of superoxide formation by the phagocytic NADPH oxidase. The beneficial effects of statins may be also related to the induction of the NRF-2/heme oxygenase-1 system and improved function of endothelial progenitor cells [231].

Pleiotropic drugs, such as nebivolol, a 3rd-generation beta-blocker, have shown beneficial effects on endothelial $^{\bullet}$NO formation [232], reversing endothelial dysfunction in patients with essential hypertension [233], improving oxidative stress [234], and preventing complications in experimental myocardial infarction [235]. Hydralazine is an old antihypertensive drug with antioxidant properties [236] and has been shown (in combination with reserperine and hydrochlorothiazide) to increase BH4 synthesis in vascular tissues [237]. Currently, hydralazine, is mainly used for the treatment of hypertension in pregnancy [238] and was repurposed for therapy of heart failure in combination of hydralazine and isosorbide dinitrate (A-HeFT trial) [239,240].

4.7. Lifestyle changes affecting eNOS function and NO/cGMP signaling

Lifestyle changes represent highly efficient and cost-effective non-pharmacological interventions for lowering cardiovascular risk [241]. While we focus here on the lifestyle factors physical exercise and intermittent fasting/caloric restriction, the list of cardiovascular benefits by healthy nutrition or smoking cessation could be extended almost endlessly (examples for effects of quitting smoking in Refs. [242,243] and for healthy nutrition effects in Refs. [244,245]). Protection by healthy diet is partly based on antioxidant components, so called nutraceuticals [246] (e.g. polyphenols [247], flavonoids [248] and NRF2 inducers such as sulforaphane [249]). Physical activity is a beneficial preventive measure against cardiometabolic diseases [250–252] and improves quality of life [253]. An inverse relationship exists between all-cause mortality and increased physical activity [254]. Physical activity reduces blood pressure dysregulation or endothelial dysfunction [255,256], while intermittent fasting decreases cardiometabolic risk markers [257,258] and lower calorie intake prevents high blood pressure and the risk of cardiovascular diseases [259]. Caloric restriction also improves cardiovascular health by decreasing blood pressure.

On a mechanistic basis, AMPK is crucial for exercise- [260] or caloric restriction (intermittent fasting)-mediated vascular protection [261–263], by suppressing inflammatory conditions and oxidative stress [264]. It acts as a fuel-sensing metabolic enzyme, phosphorylating downstream targets like peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [265], shifting the ratio of energy consumption/production for cell survival. The α1AMPK subunit, expressed in the vasculature, regulates vascular homeostasis by increasing eNOS expression and improving antioxidant mechanisms [266,267]. The protein tyrosine kinase c-Src plays a role in shear stress-dependent stimulation of eNOS in mice undergoing exercise, a protective process of physical activity that was accompanied by upregulation of aortic extracellular SOD protein expression [268]. Activation of eNOS by exercise seems to represent a fundamental mechanism by which training improves endothelial function and thereby cardiovascular health [269], also reflected by improved flow-mediated dilation by physical exercise in patients with established coronary artery disease [270] and heart failure [271,272]. Intermittent fasting activates cellular stress response elements leading to improved autophagy, suppression of apoptosis, and beneficial modification of hormonal balance [273], all of which also preserves healthy redox balance.

However, despite extensive data on the health benefits of physical activity from national and international studies [274], significant initiatives and global approaches to large clinical, controlled trials in the general population are lacking. The same applies to other beneficial factors such as healthy nutrition, intermittent fasting or quit smoking.

5. Conclusions and clinical implications

Endothelial dysfunction remains a hallmark of various cardiovascular risk factors and diseases, driven by genetic, environmental, and lifestyle influences. A central feature of this dysfunction is the oxidative and inflammatory impairment of eNOS function in both endothelial cells and perivascular adipose tissue (PVAT). While significant advances have been made in elucidating the molecular pathways involved—particularly the $^{\bullet}$NO/cGMP signaling cascade—translation into effective clinical therapies remains a challenge.

Despite current limitations, several therapeutic strategies aimed at restoring or enhancing eNOS function are showing promise. These include BH_4 supplementation, eNOS enhancers, antioxidant co-therapy, and modulation of redox-sensitive pathways. However, these approaches require further validation through well-designed, large-scale clinical trials. Similarly, interventions such as L-citrulline supplementation or cAMP-based cross-activation of the $^{\bullet}$NO/cGMP pathway have demonstrated mechanistic potential but remain underexplored

clinically.

Recent interest in the role of PVAT as a regulator of vascular homeostasis suggests that targeting eNOS function in this compartment could open new therapeutic avenues. Moreover, epigenetic regulation of eNOS and downstream signaling—though not the primary focus of this review but previously [110]—has shown encouraging results in preclinical studies and may offer long-term disease-modifying potential if translated successfully.

Nitric oxide donors, such as organic nitrates, remain a cornerstone in clinical practice, though their limitations in chronic use underscore the need for alternative strategies. Dietary and pharmacological inorganic nitrate and nitrite supplementation represent attractive, accessible options with growing evidence for cardiovascular benefit in early-phase trials. Additionally, pharmacological agents that modulate cGMP metabolism—such as sGC stimulators and phosphodiesterase inhibitors—are among the most clinically advanced approaches, with expanding therapeutic potential.

In conclusion, while the clinical translation of eNOS-targeted strategies is still in its early stages, growing mechanistic insights and emerging pharmacological tools provide a strong foundation. Future research should prioritize integrative, multimodal strategies that combine eNOS modulation with redox balance, metabolic support, and individualized therapy to harness the full therapeutic potential of the $^{\bullet}$NO/cGMP axis in cardiovascular disease.

CRediT authorship contribution statement

Huige Li: Writing – review & editing, Writing – original draft, Conceptualization. **Ulrich Förstermann:** Writing – review & editing, Writing – original draft. **Ning Xia:** Writing – review & editing, Writing – original draft. **Marin Kuntic:** Writing – review & editing, Writing – original draft. **Thomas Münzel:** Writing – review & editing, Writing – original draft. **Andreas Daiber:** Writing – review & editing, Writing – original draft, Conceptualization.

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Declaration of competing interest

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