

Implication of eNOS Uncoupling in Cardiovascular Disease

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ABSTRACT | Under physiological conditions, nitric oxide (NO) is produced in the vasculature mainly by the endothelial nitric oxide synthase (eNOS). Endothelial NO relaxes blood vessels, inhibits platelet activity, and protects against atherosclerosis. Under pathological conditions such as hypertension, diabetes, and hypercholesterolemia, eNOS may become uncoupled. Uncoupled eNOS generates superoxide at the expense of NO and contributes substantially to oxidative stress and endothelial dysfunction. Major mechanisms of eNOS uncoupling include deficiency of the eNOS cofactor tetrahydrobiopterin, deficiency of the eNOS substrate L-arginine, and eNOS S-glutathionylation. Reversal of eNOS uncoupling may represent a feasible strategy for the prevention and treatment of cardiovascular diseases.

KEYWORDS | Cardiovascular disease; eNOS uncoupling; Nitric oxide; Oxidative stress; Reactive oxygen species

ABBREVIATIONS | ACE, angiotensin-converting enzyme; ApoE-KO, apolipoprotein E-knockout; ARB, angiotensin AT1 receptor blocker; BH2, 7,8-dihydrobiopterin; BH4, tetrahydrobiopterin; CAD, coronary artery disease; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; PETN, pentaerythritol tetranitrate; ROS, reactive oxygen species; STZ, streptozotocin

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1. THE PHENOMENON OF eNOS UNCOUPLING

Under physiological conditions, nitric oxide (NO) is produced in the blood vessel mainly by the endothelial NO synthase (eNOS) [1–3]. Endothelial NO processes multiple vasoprotective properties, including vasodilation, inhibition of platelet aggregation and adhesion, and anti-atherosclerotic effects [1–3]. In addition, eNOS-derived NO has antioxidant activities by abating Fenton-type reactions, terminating radical chain reactions, and inhibiting peroxidases and oxidases through S-nitrosylation of allosteric thiols [4].

Under pathological conditions associated with oxidative stress, however, eNOS may become dysfunctional, producing superoxide at the expense of NO. This phenomenon is referred to as eNOS uncoupling [5, 6]. Uncoupling of eNOS is not an all-or-none phenomenon. Rather, uncoupled and coupled eNOS proteins may exist in the same cell at the same time [6, 7], as shown in the hypercholesterolemic apolipoprotein E-knockout (ApoE-KO) mice [8]. In this murine model of atherosclerosis, the protective role of NO derived from coupled eNOS overwhelms the detrimental effect of superoxide produced by uncoupled eNOS [8]. This may be an explanation for the observations that genetic deletion of eNOS [8–10] and pharmacological inhibition [11] of (both the coupled and the uncoupled) eNOS accelerate atherosclerosis development, despite the existence of eNOS uncoupling in these animals.

2. MOLECULAR MECHANISMS OF eNOS UNCOUPLING

Numerous mechanisms have been implicated in eNOS uncoupling [2, 3, 12]. Among these, depletion of tetrahydrobiopterin (BH4), an essential cofactor for the eNOS enzyme, is likely to play a major role in eNOS uncoupling and endothelial dysfunction. Peroxynitrite and superoxide can oxidize BH4 to 7,8-dihydrobiopterin (BH2), leading to BH4 deficiency [13]. BH2 competes with BH4 for eNOS binding, but has no cofactor activity. BH2 can be reduced back to BH4 by the enzyme dihydrofolate reductase (DHFR) [1, 2].

Another important cause of eNOS uncoupling is a deficiency of eNOS substrate L-arginine, mostly due

to upregulation of arginase expression/activity. Arginases metabolize L-arginine to urea and L-ornithine [14]. The expression/activity of vascular arginases is enhanced by diverse stimuli [15], including angiotensin II [16], high glucose [17], thrombin [18], oxidative species [19], and oxidized low-density lipoprotein (oxLDL) [20]. An increased arginase expression/activity decreases L-arginine bioavailability for eNOS, and can lead to eNOS uncoupling.

Recently, S-glutathionylation has been identified as another crucial mechanism for eNOS uncoupling [21]. S-Glutathionylation is a posttranslational modification in which a glutathione tripeptide is reversibly bound to the protein via the formation of a disulfide bond with a protein thiol [12]. S-Glutathionylation of cysteine residues in the reductase domain (Cys689 and Cys908) [21] shifts eNOS from an NO-generating enzyme to a superoxide producer. This mechanism has been implicated in eNOS uncoupling under conditions of aging [22], ambient ultrafine particles exposure [23], organic nitrate-induced endothelial dysfunction [24, 25], angiotensin II-induced vascular dysfunction [26–28], in vessels of hypertension rats [21], in hyperglycemia and experimental diabetes [29, 30] and in response to carbamylated LDL [31]. It is noteworthy that eNOS uncoupling induced by BH4 deficiency and by S-glutathionylation is mechanistically independent of each other. However, they are functionally linked and act in concert to regulate NO or superoxide production by eNOS [32]. BH4 deficiency leads to superoxide production by the oxygenase domain which in turn decreases the ratio of reduced form of glutathione (GSH) to glutathione disulfide (GSSG) and thereby initiates eNOS S-glutathionylation and eNOS uncoupling. Vice versa, S-glutathionylation triggers superoxide production from the reductase domain which then oxidizes BH4, and thus results in the superoxide production from the oxygenase domain [32].

The consequences of eNOS uncoupling are reduced NO production and augmentation of pre-existing oxidative stress by overproduction of reactive oxygen species (ROS) such as superoxide and subsequently peroxynitrite. This potentiation then leads to enhanced oxidation of BH4, upregulation of arginase expression/activity, and S-glutathionylation of eNOS, creating a vicious circle (**Figure 1**). Hence, eNOS uncoupling is a key mechanism in and con-

tributes substantially to endothelial dysfunction and cardiovascular disease.

3. UNCOUPLING OF eNOS IN CARDIOVASCULAR DISEASE

All established cardiovascular risk factors, such as hypertension, hypercholesterolemia, and diabetes mellitus, enhance oxidative stress and induce eNOS uncoupling [6, 33].

3.1. Hypertension

Uncoupled eNOS contributes substantially to vascular oxidative stress in hypertension [6, 33]. BH4 deficiency, L-arginine deficiency, and S-glutathionylation have been shown as molecular mechanisms for eNOS uncoupling in animal models of hypertension, including angiotensin II-induced hypertension, spontaneously hypertensive rats (an animal model of genetic hypertension) and deoxycorticosterone acetate-salt (DOCA-salt) hypertension. The deficiency of BH4 has been attributed to NADPH oxidase-mediated of BH4 oxidation [34] and to reduced BH4 recycling from BH2 due to a downregulation of endothelial dihydrofolate reductase (DHFR) [35]. L-Arginine deficiency in hypertension models is likely to result from an upregulation of arginase expression/activity in blood vessels [36–38]. Uncoupling of eNOS by S-glutathionylation is evident in angiotensin II-induced hypertension [26, 27] and in vessels of hypertension rats [21]. Reversal of eNOS uncoupling reduces blood pressure in hypertensive animals [39] or contributes to blood pressure reduction by some antihypertensive drugs [28].

In hypertensive patients, intra-arterial infusion of BH4 augments forearm blood flow response to acetylcholine [40]. Oral BH4 administration improves endothelial function and reduces blood pressure in human subjects with essential hypertension [41], indicating the relevance of BH4 deficiency and eNOS uncoupling in human hypertension.

3.2. Hypercholesterolemia

Both native LDL and oxLDL have been shown to stimulate superoxide/peroxynitrite production, and to uncouple eNOS [42, 43]. ROS production from un-

coupled eNOS has been shown in LDL-treated endothelial cells, in hypercholesterolemic ApoE-KO mice [44], and in hypercholesterolemic patients [45]. Hypercholesterolemia leads to BH4 oxidation and BH4 deficiency [44]. In addition, L-arginine deficiency also represents a cause of eNOS uncoupling in hypercholesterolemia. An upregulation of arginase expression and/or activity has been shown in ApoE-KO mice [46, 47] and in hyperlipidemic rabbits [48]. The aortic arginase activity in ApoE-KO mice is significantly reduced after the removal of the endothelium, suggesting an important contribution of endothelial cells [46]. The functional relevance of arginase upregulation in atherosclerosis has been shown in ApoE-KO mice. Selective endothelial overexpression of arginase 2 induces endothelial dysfunction and enhances atherosclerosis in mice [49]. Treatment with an arginase inhibitor for 4 or 8 weeks reduces aortic plaque burden in ApoE-KO mice [46].

Vascular (but not plasma) BH4 content has been shown to be an important determinant of eNOS uncoupling and superoxide production in vessels isolated from patients with coronary artery disease (CAD) [50]. BH4 restores endothelial function in patients with hypercholesterolemia [45]. Serum levels of carbamylated LDL are elevated in patients with CAD [51, 52] and carbamylated LDL-induced eNOS uncoupling by S-glutathionylation has been recently proposed to be a molecular mechanism contributing to the pathogenesis of atherosclerosis [31].

3.3. Diabetes Mellitus

Uncoupling of eNOS has been observed in streptozotocin (STZ)-induced type 1 diabetes mellitus [53]. The underlying mechanisms involve NADPH oxidase-mediated BH4 oxidation. Indeed, BH4 oxidation and BH4 deficiency are evident in STZ-treated mice [54] and rats [55]. In addition, diabetes also causes BH4 deficiency by reducing BH4 synthesis. Enhanced ROS production in diabetes accelerates proteasomal degradation of guanosine 5'-triphosphate cyclohydrolase 1 (GCH1), a rate-limiting enzyme in the biosynthesis of BH4 [29, 56, 57]. Moreover, eNOS S-glutathionylation represents another important mechanism of eNOS uncoupling in the setting of type 1 diabetes [29].

In mouse models of type 2 diabetes, a relative BH4 deficiency is evident due to enhanced BH4 oxidation and a low BH4:BH2 ratio [58–60]. The increased

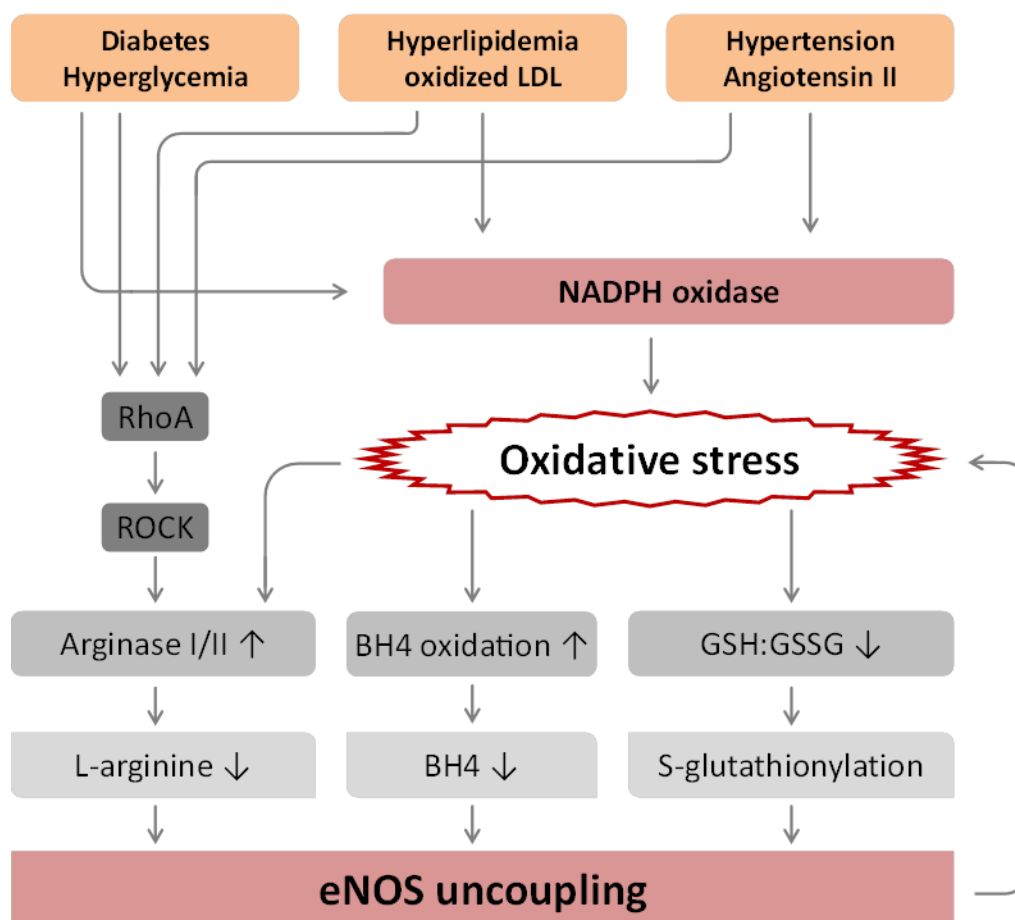


FIGURE 1. Uncoupling of eNOS in cardiovascular disease. Cardiovascular risk factors such as hypertension, hypercholesterolemia and diabetes mellitus promote superoxide production by eNOS (eNOS uncoupling) through three major mechanisms: depletion of the eNOS cofactor tetrahydrobiopterin (BH4), depletion of the eNOS substrate L-arginine, and eNOS S-glutathionylation. NADPH oxidase-derived reactive oxygen species (e.g., superoxide and subsequently peroxynitrite) oxidize BH4 leading to BH4 deficiency. L-Arginine deficiency is caused by upregulation of arginase expression/activity. Oxidative stress-induced reduction in GSH:GSSG ratio favors eNOS S-glutathionylation. Uncoupled eNOS produces reactive oxygen species, which in turn oxidize the BH4, increase arginase expression and activity, and enhance eNOS S-glutathionylation, creating a vicious circle. GSH and GSSG denote reduced form of glutathione and glutathione disulfide, respectively.

levels of angiotensin II in diabetic patients may additionally reduce DHFR expression and decrease BH4 recycling from BH2 [35].

L-Arginine deficiency and eNOS uncoupling have also been documented in rodent models of type 1 [17, 61–63] as well as type 2 diabetes [64]. High glucose and persistent insulin stimulation upregulate arginase expression in endothelial cells [17, 62, 65].

In patients with type 2 diabetes mellitus, the reduced forearm blood flow response to acetylcholine is significantly improved by BH4, an effect that can be blocked by NOS inhibition [66]. In contrast, BH4 has no effect in healthy controls [66]. These results indicate that BH4 deficiency and eNOS uncoupling play a role in diabetes mellitus-induced vascular dysfunction.

Plasma arginase activity is elevated in patients with type 2 diabetes mellitus [67]. An upregulation of arginase 1 in coronary arterioles of patients with (type 1 or type 2) diabetes mellitus has been shown to contribute to the reduced NO production and consequently diminished vasodilation [68]. Arginase inhibition markedly improves endothelium-dependent vasodilation in the forearm of patients with type 2 diabetes mellitus and CAD whereas it does not affect endothelial function in healthy controls [69]. This observation indicates a functional role of arginase contributing to endothelial dysfunction in patients with diabetes.

4. THERAPEUTIC STRATEGIES

Uncoupling of eNOS plays a crucial role in endothelial dysfunction. On this account, it is an important objective of cardiovascular disease treatment to prevent eNOS uncoupling or to reverse an existing eNOS uncoupling. Since molecular mechanisms underlying eNOS uncoupling are more and more understood, various pharmacological approaches, which aim at the prevention of eNOS uncoupling, have been successfully studied in animal models [6, 7]. Examples are angiotensin-converting enzyme (ACE) inhibitors, angiotensin AT1-receptor blockers (ARBs), the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), the organic nitrate pentaerythritol tetranitrate (PETN), and the plant polyphenolic phytoalexin resveratrol (for details see our recent review articles [6, 7, 33]). These compounds prevent BH4 oxidation partly by inhibiting NADPH oxidase expression or activity. Some of these compounds, such as ARB and PETN, also upregulate DHFR and hereby enhance BH4 regeneration from BH2 [6, 7]. Others, such as statins [17, 18, 70, 71], ACE inhibitors [72], and ARBs [16], additionally inhibit arginase activity which in turn leads to an improved eNOS functionality. It has been demonstrated that ACE inhibitors [28], ARBs [24], and PETN [29] additionally prevent eNOS S-glutathionylation. The enhanced NO bioavailability, which comes along with these treatment approaches, is part of the pleiotropic effects that contribute to their therapeutic benefit.

The health impact of long-term L-arginine supplementation is currently under debate [14]. Two clinical studies have shown that chronic L-arginine

supplementation is not beneficial and can even be potentially harmful [73, 74]. In contrast, small-scale “proof-of-concept” clinical studies have shown that local administration of arginase inhibitors improves vascular function in aged humans [75] as well as in patients with CAD and type 2 diabetes mellitus [69], heart failure [76], and hypertension [77]. Larger clinical studies with systemic arginase inhibition are warranted [15, 78].

5. CONCLUSION

Uncoupling of eNOS represents a major mechanism for the reduced NO production, enhanced oxidative stress, and endothelial dysfunction in cardiovascular disease. Reversal of eNOS uncoupling may represent a feasible strategy for the prevention and treatment of cardiovascular diseases.

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