Free radical production by dysfunctional eNOS

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ENDOTHELIAL NOS

Endothelial NOS is a dimeric, bidomain enzyme consisting of a C-terminal reductase domain which binds NADPH, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), an N-terminal oxidase domain which binds a prosthetic heme group, tetrahydrobiopterin (BH4), oxygen, and L-arginine and a regulatory calmodulin binding sequence. Under physiological conditions, after binding of Ca²⁺/calmodulin between the oxygenase and reductase domain, electrons are donated by reduced NADPH and shuttled through the reduced flavins toward the oxidase domain. At the heme site molecular oxygen is reduced and incorporated into L-arginine to form NO and L-citrulline. The essential cofactor BH4 has been shown to be a key factor in eNOS catalysis. Experiments in purified eNOS showed that in the absence of BH4, “eNOS uncoupling” may occur—that is, uncoupling of NADPH oxidation and NO synthesis, with oxygen instead of L-arginine as terminal electron acceptor, resulting in the formation of superoxide. The crucial role of BH4 in eNOS coupling was supported by studies in both endothelial cells as well as isolated vessels showing that reduction of intracellular BH4 concentrations by inhibition of GTP cyclohydrolase I, the rate limiting enzyme for BH4 synthesis, resulted in a reduction of NO synthesis and enhanced superoxide generation, which could be reversed by incubation with sepiapterin, substrate for BH4 synthesis. Importantly, several clinical studies have demonstrated beneficial effects of BH4 administration on endothelial function in patients with cardiovascular risk factors, such as hypercholesterolaemia, smoking, hypertension, and diabetes or coronary artery disease. The underlying reason for the decreased BH4 bioavailability in endothelial dysfunction has not been fully elucidated but may be related to impaired synthesis, decreased affinity of eNOS for its cofactor, or increased catabolism. Biosynthesis of BH4 occurs either via a de novo pathway in which the enzyme GTP cyclohydrolase I is the rate limiting step, or via a so-called salvage pathway that utilises sepiapterin as an intermediate step. Rapid depletion of BH4 in the vessel wall following pharmacological inhibition of GTP cyclohydrolase I suggests that BH4 turnover in the endothelium is relatively high. Both depletion of GTP as well as down regulation of the expression of GTP cyclohydrolase I have been postulated to contribute to the reduced BH4 bioavailability in endothelial dysfunction. Recently, BH4 was shown to be a major target for oxidation by peroxynitrite suggesting that enhanced BH4 catabolism caused by prolonged oxidative stress may be an important underlying reason for the decreased BH4 bioavailability in endothelial dysfunction. Indeed, in hypertensive vessels BH4 oxidation could be demonstrated, leading to...

Abbreviations: BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide
eNOS uncoupling with reduced formation of NO and increased superoxide production.\textsuperscript{10} It was hypothesised that the ratio between reduced and oxidised BH4 metabolites tightly controls superoxide formation from eNOS.\textsuperscript{12} Consistently, the antioxidant vitamin C has been shown to potentiate eNOS enzymatic activity by protecting BH4 from oxidation through its chemical stabilisation.\textsuperscript{13}

**IMPROVING ENDOTHELIAL FUNCTION**

The above data indicate that reduced BH4 bioavailability may play a key role in the aetiology of endothelial dysfunction associated with conditions such as hypercholesterolaemia, hypertension, diabetes, and atherosclerosis. Administration of BH4 or manipulating vascular BH4 status by either enhancing BH4 synthesis or preventing its oxidation may be promising strategies for improving endothelial function and reducing cardiovascular risk. Thus far, several clinical trials have shown beneficial effects of short term BH4 supplementation on endothelial function.\textsuperscript{14} Recently, we have demonstrated that BH4-dependent stimulatory effect on NO mediated function in vivo has been shown exert a BH4 dependent stimulatory effect on NO mediated endothelial function.\textsuperscript{15} Interestingly, it was recently reported that statins, in addition to augmenting eNOS expression, may also potentiate GTP cyclohydrolase I gene expression and BH4 synthesis, thereby improving eNOS function.\textsuperscript{16}

**CONCLUSION**

Normal endothelial NO synthase function is of fundamental importance for vascular homeostasis. In many vascular diseases “eNOS uncoupling” appears to be present, leading to increased superoxide and reduced NO production. The essential eNOS cofactor BH4 has a crucial role in maintaining eNOS in the optimal “coupled” state. In various vascular disease conditions loss of BH4 bioavailability and subsequent eNOS uncoupling may contribute to impaired NO mediated endothelial function. Mechanisms that modulate BH4 status in human vascular disease represent promising targets for therapeutic interventions aimed at prevention of atherosclerotic disease.

**REFERENCES**

1. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 2000;101:1899–906.
2. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–6.
3. Xia Y, Tao AL, Berk V, et al. Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. J Biol Chem 1998;273:25804–8.
4. Shi W, Wang X, Shih DM, et al. Paradoxical reduction of fatty streak formation in mice lacking endothelial nitric oxide synthase. Circulation 2002;105:2078–82.
5. Ozaki M, Kowashima S, Yamashita T, et al. Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apolipoprotein-deficient mice. J Clin Invest 2002;110:331–40.
6. Wever RM, van Dam T, van Rijn HM, et al. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochem Biophys Res Commun 1997;237:340–4.
7. Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. Cirrhosis 1995;91:139–44.
8. Stroes ES, Kastelein PJ, Cosentino F, et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 1997;99:41–6.
9. Heitzer T, Brockhoff C, Mayer B, et al. Tetrahydrobiopterin improves endothelial-dependent vasodilation in chronic smokers: evidence for a dysfunctional nitric oxide synthase. Circ Res 2000;86:536–41.
10. Kuzkaya N, Weissmann N, Harrison DG, et al. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem 2003;278:22546–54.
11. Landmesser U, Dikalov S, Price SR, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 2003;111:1201–9.
12. Vasquez-Vivar J, Martasek P, Whitsett J, et al. The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR trapping study. Biochem J 2002;362:733–9.
13. Heller R, Unbehauen A, Schellenberg B, et al. L-ascorbic acid potentiates endothelial nitric oxide synthase via a chemical stabilization of tetrahydrobiopterin. J Biol Chem 2001;276:40–7.
14. D’Uscio LV, Milisien S, Richardson D, et al. Long-term vitamin C treatment increases vascular tetrahydrobiopterin levels and nitric oxide synthase activity. Circ Res 2003;92:88–95.
15. Verhaar MC, Stroes E, Rabelink TJ. Follates and cardiovascular disease. Arterioscler Thromb Vasc Biol 2002;22:6–13.
16. Hattori Y, Nakanoishi N, Akimoto K, et al. HMGC-a reductase inhibitor increases GTP cyclohydrolase I mRNA and tetrahydrobiopterin in vascular endothelial cells. Arterioscler Thromb Vasc Biol 2003;23:176–82.