Free radical production by dysfunctional eNOS

M C Verhaar, P E Westerweel, A J van Zonneveld, T J Rabelink

Endothelial derived nitric oxide (NO) plays a major role in cardiovascular homeostasis. It has important anti-atherosclerotic properties which include regulation of vasomotor tone and vessel wall permeability, suppression of leucocyte adhesion to the endothelial surface, inhibition of platelet aggregation, and inhibition of vascular smooth muscle cell migration and proliferation. The important role of NO in the cardiovascular system is highlighted by key observations in animal models. Inhibition of endothelial NO synthase (eNOS), the enzyme that catalyses endothelial NO synthesis, accelerates atherogenesis. Similarly, genetic deletion of eNOS in mice leads to hypertension, defective vascular remodelling, vascular thrombosis, and enhanced leucocyte–endothelial cell interactions. In humans, all major cardiovascular risk factors, including hypercholesterolaemia, hypertension, diabetes, and smoking, have been associated with endothelial dysfunction, characterised by impaired NO bioavailability. Importantly, the impairment of NO mediated endothelial function is an independent predictor of adverse cardiac events.\(^1\) Taken together, current data strongly suggest that impaired NO activity is a crucial factor in the pathogenesis of cardiovascular disease. Improving endothelial NO bioavailability in vivo may reduce cardiovascular risk and has emerged as a major therapeutic goal.

**SYNTHESIS AND DEGRADATION OF NITRIC OXIDE**

In vivo NO bioactivity is determined by the balance between synthesis and degradation of NO. The biosynthesis of endothelial NO is catalysed by the enzyme eNOS and requires the amino acid L-arginine, nicotinamide adenine dinucleotide phosphate (NADPH), and molecular oxygen as substrates, as well as several cofactors and prosthetic groups.

Inactivation of NO may occur by its reaction with oxyhaemoglobin in erythrocytes but also by reacting with superoxide anions, resulting in the formation of peroxynitrite. Increased superoxide production is a feature of various vascular disease states and is now recognised as an important determinant of impaired NO activity in vivo.\(^2\) Potential sources of superoxide in the vessel wall include mitochondria, cytochrome P450-type enzymes, cyclooxygenase, lipoxygenase, NAD(P)(H) oxidases, and xanthine oxidase. Interestingly, in vitro studies in purified eNOS have shown that eNOS itself, beside its key role in NO production, may be a potential source of superoxide.\(^3\) Accordingly, in animal models of atherosclerosis removal of the endothelial monolayer or infusion of a selective NO synthase antagonist not only prevented NO formation but also inhibited increased formation of superoxide. Recent reports, demonstrating that diet induced fatty streak formation was paradoxically reduced in mice lacking eNOS whereas chronic overexpression of eNOS accelerated atherosclerotic lesion formation in apoE-deficient mice,\(^4\) provide in vivo support for such a dual role of eNOS and identify eNOS (dys)function rather than eNOS expression as a key target for anti-atherosclerotic therapies.

**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 Ca\(^{2+}\)/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.\(^5\) 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.\(^6\) 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.\(^7\)\(^8\) Thus, in patients at increased cardiovascular risk, the decreased availability of BH4 in the vessel wall with subsequent eNOS dysfunction may be a crucial determinant of impaired NO mediated endothelial function.

**BH4 BIOAVAILABILITY**

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.\(^9\) Indeed, in hypertensive vessels BH4 oxidation could be demonstrated, leading to impaired NO bioactivity.

Abbreviations: BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide
endothelial function. Mechanisms that modulate BH4 status and/or eNOS uncoupling may contribute to impaired NO mediated vasodilation and disease conditions loss of BH4 bioavailability and subsequent eNOS in the optimal "coupled" state. In various vascular diseases "eNOS uncoupling" appears to be present, leading to increased superoxide production. It was hypothesised that the ratio between reduced and oxidised BH4 metabolites tightly controls superoxide formation from eNOS. Consistently, the antioxidant vitamin C has been shown to potentiate eNOS enzymatic activity by protecting BH4 from oxidation through its chemical stabilisation.

**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 hypercholesterolemia, 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. However, its potential as long term clinical strategy is questionable. BH4 in its active, reduced form is highly unstable and therefore not suitable for oral administration. Furthermore, administration of BH4 in the presence of conditions with increased oxidative stress may lead to rapid oxidative degradation of BH4 and thus limited duration of the beneficial effects. Supplementation of vitamin C, which has been shown to prevent oxidation of BH4, or a combination of vitamin C and BH4 may prove more useful therapeutic strategies. Indeed, long term vitamin C administration in vivo has been shown to exert a BH4 dependent stimulatory effect on NO mediated endothelial function. Recently, we have demonstrated that folate is also capable of BH4 dependent potentiation of eNOS function in vitro and improving NO mediated endothelial function in vivo. The exact mechanisms are not clear but may involve BH4 stabilisation, stimulation of BH4 regeneration from the inactive oxidised form, enhanced binding of BH4 to eNOS, or interaction with the active site of eNOS mimicking BH4. 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.

**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.

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