

invited review

Vascular endothelial dysfunction: does tetrahydrobiopterin play a role?

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Katusic, Zvonimir S. Vascular endothelial dysfunction: does tetrahydrobiopterin play a role? *Am J Physiol Heart Circ Physiol* 281: H981–H986, 2001.—Tetrahydrobiopterin is one of the most potent naturally occurring reducing agents and an essential cofactor required for enzymatic activity of nitric oxide synthase (NOS). The exact role of tetrahydrobiopterin in the control of NOS catalytic activity is not completely understood. Existing evidence suggests that it can act as allosteric and redox cofactors. Suboptimal concentration of tetrahydrobiopterin reduces formation of nitric oxide and favors “uncoupling” of NOS leading to NOS-mediated reduction of oxygen and formation of superoxide anions and hydrogen peroxide. Recent findings suggest that accelerated catabolism of tetrahydrobiopterin in arteries exposed to oxidative stress may contribute to pathogenesis of endothelial dysfunction present in arteries exposed to hypertension, hypercholesterolemia, diabetes, smoking, and ischemia-reperfusion. Beneficial effects of acute and chronic tetrahydrobiopterin supplementation on endothelial function have been reported in experimental animals and humans. Furthermore, it appears that beneficial effects of some antioxidants (e.g., vitamin C) on vascular function could be mediated via increased intracellular concentration of tetrahydrobiopterin. In this review, the potential role of tetrahydrobiopterin in the pathogenesis of vascular endothelial dysfunction and mechanisms underlying beneficial vascular effects of tetrahydrobiopterin will be discussed.

nitric oxide; oxidative stress; vitamin C

NITRIC OXIDE plays a key role in vascular homeostasis affecting wide range of functions, including local control of blood vessel diameter and tissue blood flow (10). Biosynthesis of nitric oxide is dependent on enzymatic activity of nitric oxide synthase (NOS). Three distinct NOS isoforms have been identified by molecular cloning: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Tetrahydrobiopterin is a cofactor essential for the catalytic activity of all three NOS isoforms (11, 23, 27, 37). During the last 10 years, significant progress has been made in understanding the role of tetrahydrobiopterin in the control of NOS function. Tetrahydrobiopterin has profound effects on the struc-

ture of NOS, including the ability to shift its heme iron to a high spin state, increase arginine binding, and stabilize the active dimeric form of the enzyme (11). There is also evidence that NOS-bound tetrahydrobiopterin may act as a redox-active cofactor, but unlike aromatic amino acid hydroxylases where the fully reduced pterin serves as a reducing agent for oxygen, NOS is not coupled to dihydropteridin reductase as a tetrahydrobiopterin-regenerating system (11). The exact redox mechanism by which tetrahydrobiopterin participates in the biosynthesis of nitric oxide is still not understood (11). However, accumulated evidence indicates that optimal concentration of tetrahydrobiopterin is of fundamental importance for normal function of eNOS and vascular endothelial cells. This review will focus on the potential role of tetrahydrobiopterin in the pathogenesis of endothelial dysfunction and vascular disease.

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ENDOTHELIAL DYSFUNCTION

Normal vascular endothelial cells support cardiovascular function by promoting vasodilatation and by inhibiting platelet aggregation, white blood cell adhesion, and smooth muscle cell proliferation. In contrast, dysfunctional endothelium promotes vasoconstriction, favors platelet aggregation, white blood cell adhesion, and smooth muscle cell proliferation. It is now well established that the endothelium becomes dysfunctional in arteries chronically exposed to cardiovascular risk factors. Hypercholesterolemia, hyperglycemia, hypertension, and smoking are the most common risks associated with endothelial dysfunction. Although the molecular basis of endothelial dysfunction is not completely understood, numerous studies point to the loss of nitric oxide biological activity and/or biosynthesis as a central mechanism (9). Restoration of normal nitric oxide levels in diseased arteries is a major therapeutic goal and could be achieved by several different classes of drugs, including nitric oxide donors, L-arginine, statins, angiotensin-converting enzyme inhibitors, antioxidants, and estrogen replacement. More recently, eNOS gene transfer technology has also been employed in attempts to normalize endothelial function in diseased arteries (3). Realization that availability of tetrahydrobiopterin may also affect nitric oxide production in endothelial cells provided the rationale for supplementation with exogenous tetrahydrobiopterin. During the last couple of years, successful restoration of endothelial function by short-term administration of tetrahydrobiopterin has been achieved in patients with hypercholesterolemia and atherosclerosis, and in smokers (13, 25, 35, 42). Although the exact mechanism underlying the beneficial effect of tetrahydrobiopterin is still unknown, the most likely explanation is increased production of nitric oxide due to activation of eNOS.

In the early 1990s biochemical studies demonstrated that in the presence of suboptimal concentrations of tetrahydrobiopterin, activation of nNOS leads to "uncoupling of NOS" with subsequent increased formation of superoxide anions and hydrogen peroxide (12, 30). These findings have been confirmed and extended to eNOS (43, 46, 47), suggesting that in endothelial cells consumption of NADPH can become uncoupled from nitric oxide synthesis, resulting in the production of superoxide anions and hydrogen peroxide (Fig. 1). It is important to note that a series of *in vitro* biochemical studies demonstrated that eNOS is the most "tightly coupled" of the NOS isoforms (26), implying that nNOS and iNOS are potentially more powerful sources of reactive oxygen species. Definitive *in vivo* evidence that "uncoupling" of eNOS is an important mechanism of endothelial dysfunction is missing. However, this hypothesis continues to attract attention of vascular biologists. At the present time, it is generally accepted that reduced availability of tetrahydrobiopterin causes reduction in nitric oxide production, and that enzymatic activity of "uncoupled" NOS could be a source of reactive oxygen species. It appears that supplementa-

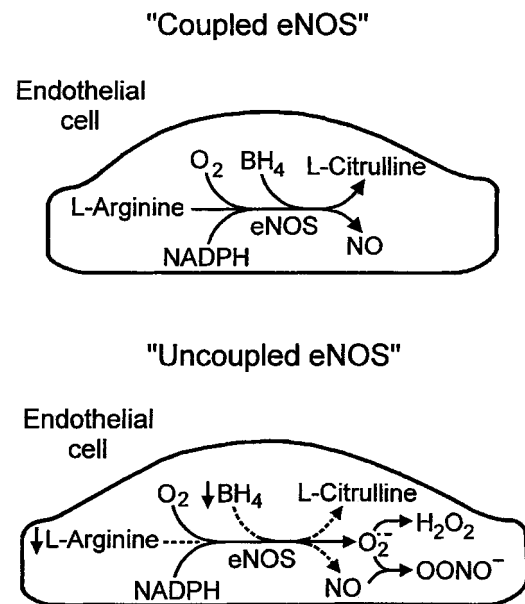


Fig. 1. Schematic representation of "uncoupling" of nitric oxide (NO) synthesis from consumption of NADPH. Suboptimal concentrations (\downarrow) of L-arginine and/or tetrahydrobiopterin (BH₄) are required for "uncoupling." O₂⁻, superoxide anion; H₂O₂, hydrogen peroxide; OONO⁻, peroxynitrite anion; eNOS, endothelial NO synthase.

tion with exogenous tetrahydrobiopterin can restore normal nitric oxide biosynthesis by providing optimal conditions for NOS catalytic activity.

Early studies on cultured endothelial cells demonstrated that indeed, an increase in intracellular tetrahydrobiopterin levels stimulate eNOS activity (45). Interestingly, a study by Rosenkranz-Weiss et al. (31) demonstrated that tetrahydrobiopterin levels are significantly higher in freshly isolated endothelial cells than in cultured endothelial cells. Culturing endothelial cells causes loss of tetrahydrobiopterin. The mechanism responsible for the loss of tetrahydrobiopterin is not completely understood, but it is known that in the cell culture tetrahydrobiopterin efflux dominates over cell retention (11). This finding suggests that results obtained with cultured endothelial cells may not be representative of the changes in tetrahydrobiopterin metabolism that occur in intact arteries. Our measurements of tetrahydrobiopterin levels in normal arteries demonstrated that more than 60% of total tetrahydrobiopterin is present in endothelial cells (40), revealing that a single layer of endothelial cells has a higher content of tetrahydrobiopterin than media and adventitia together. High capacity of endothelium to synthesize tetrahydrobiopterin most likely reflects an important role of this pterin in regulation of endothelial function. Pharmacological or genetic manipulation of tetrahydrobiopterin availability provided initial insight into possible consequences of tetrahydrobiopterin deficiency on endothelial function. Studies on isolated aorta, coronary, and cerebral arteries demonstrated that inhibition of tetrahydrobiopterin production causes alterations or impairment of endothelial function (6, 7, 20). Similar observations were made in coronary microcirculation (39). These findings were in

agreement with results obtained in experiments with cultured endothelial cells, and they supported the idea that availability of tetrahydrobiopterin in endothelial cells may affect NOS activity and ultimately the production of nitric oxide. Obviously, the next step required better understanding of tetrahydrobiopterin biosynthesis in normal and diseased blood vessels.

Metabolism of tetrahydrobiopterin in the vascular wall has not been systematically studied. Very little is known about catabolism of tetrahydrobiopterin in living cells. Turnover of tetrahydrobiopterin in blood vessels appears to be very rapid. In isolated canine basilar arteries 6 h of incubation with GTP cyclohydrolase I inhibitor DAHP resulted in 95% depletion of intracellular tetrahydrobiopterin (20). This raised the possibility that excessive oxidation of tetrahydrobiopterin may contribute to the pathogenesis of endothelial dysfunction. In 1997 we proposed that tetrahydrobiopterin could be an important molecular target for oxidative stress (21). During the last four years, several groups, including our own, have tested this hypothesis and considerable progress has been made.

EFFECT OF OXIDATIVE STRESS ON TETRAHYDROBIOPTERIN METABOLISM

Tetrahydrobiopterin is one of the most potent naturally occurring reducing agents. It is therefore reasonable to hypothesize that oxidative stress may lead to excessive oxidation and depletion of tetrahydrobiopterin. Indeed, peroxyxynitrite does oxidize tetrahydrobiopterin under *in vitro* conditions (24, 28). Laursen et al. (24) provided the first evidence that in intact arteries oxidation of tetrahydrobiopterin by peroxyxynitrite may have important implications for the pathogenesis of endothelial dysfunction. Their findings strongly support the idea that tetrahydrobiopterin is a molecular target for oxidative stress and that oxidation of tetrahydrobiopterin may cause "uncoupling" of eNOS. Consistent with these findings, recent studies demonstrated that vitamin C stimulates NOS via chemical stabilization of tetrahydrobiopterin in cultured human umbilical vein endothelial cells (14, 16). This effect of vitamin C appears to be independent of the chemical antagonism between vitamin C and superoxide anions (2). The chemical nature of oxidants responsible for oxidation of tetrahydrobiopterin is unclear and remains to be determined. These *in vitro* studies reinforced the concept that in vascular endothelial cells, prooxidant conditions can accelerate catabolism of tetrahydrobiopterin. Most importantly, these findings could help to explain the mechanisms underlying the beneficial effect of vitamin C (and possibly some other antioxidants) on vascular endothelial function. However, it is important to keep in mind that protection of tetrahydrobiopterin with vitamin C has not been demonstrated *in vivo*. Our preliminary findings indicate an increased dietary intake of vitamin C prevents development of endothelial dysfunction in aortas and carotid arteries of ApoE-deficient mice, an experimental model of human hypercholesterolemia and atherosclerosis

(d'Uscio and Katusic, unpublished observation). Whether this *in vivo* effect of vitamin C is related to its ability to protect tetrahydrobiopterin is currently under investigation.

Very little information is available concerning tetrahydrobiopterin levels in diseased blood vessels. In 1998, Cosentino et al. (8) did not detect any difference in tetrahydrobiopterin levels between control aortas and aortas obtained from prehypertensive rats. Similarly, the tetrahydrobiopterin level was normal in pre-eclamptic placentas (22). High fructose diet (model of insulin resistance) caused modest reduction (~10%) of the rat aortic tetrahydrobiopterin (33, 34), suggesting that endothelial dysfunction in rats with insulin resistance could be due to alterations in tetrahydrobiopterin metabolism. In contrast, hypercholesterolemia and atherosclerosis are associated with increased levels of tetrahydrobiopterin in aortas of ApoE-deficient mice or rabbits fed a high cholesterol diet (unpublished observations). Elevated levels of neopterin (a byproduct of tetrahydrobiopterin biosynthesis) were also detected in plasma of patients with atherosclerosis (36). This is consistent with the reported ability of proinflammatory cytokines to upregulate expression and enzymatic activity of GTP cyclohydrolase I (rate-limiting enzyme in biosynthesis of tetrahydrobiopterin) in vascular endothelial cells (19, 31). Thus, despite the fact that there is very little evidence for the loss of tetrahydrobiopterin from the diseased blood vessel wall, endothelial function was normalized by *in vitro* tetrahydrobiopterin supplementation in experimental animals with insulin resistance and hypercholesterolemia (24, 33, 34, 38). The exact mechanisms responsible for the beneficial effects of tetrahydrobiopterin is unclear but most likely involves increased enzymatic activity of eNOS and/or antioxidant activity of tetrahydrobiopterin. Indeed, as mentioned earlier in this review, several groups (2, 14, 16) demonstrated that in cultured endothelial cells, increased availability of tetrahydrobiopterin could account for stimulation of eNOS activity induced by vitamin C. Most importantly, oral supplementation for 8 wk with tetrahydrobiopterin increased eNOS activity and reduced superoxide anion formation by eNOS in the aortas of insulin-resistant rats (34), providing strong evidence for the important role of tetrahydrobiopterin in the pathogenesis of endothelial dysfunction.

Beneficial effects of tetrahydrobiopterin supplementation on vascular endothelial function of experimental animals and humans have been reported by a number of laboratories (Table 1). However, it is interesting that despite the heterogeneity of animal models and patient populations studied, tetrahydrobiopterin consistently improved endothelial function in all of the reported studies. The explanation for the beneficial effect of tetrahydrobiopterin could be due to the fact that oxidative stress is the most likely common mechanism underlying endothelial dysfunction in conditions like hypercholesterolemia, diabetes, and smoking. If tetrahydrobiopterin is a common molecular target for oxidative stress, then it is conceivable that supplementa-

Table 1. *Beneficial effect of tetrahydrobiopterin supplementation on endothelial dysfunction*

Disease	Species	Vascular Bed	Reference
Atherosclerosis	Human	Coronary artery	25
Atherosclerosis	Human	Coronary arterioles	38
Atherosclerosis	Human	Saphenous vein	44
Atherosclerosis	Pig	Coronary arterioles	38
Diabetes	Rat	Aorta	29, 33, 34, 48
Hypercholesterolemia	Human	Forearm	35
Hypertension	Rat	Aorta	8
Ischemia	Rat	Kidney	18
Ischemia-reperfusion	Pig	Coronary artery	39
Ischemia-reperfusion	Pig	Lung	32
Smoking	Human	Saphenous vein	15
Smoking	Human	Brachial artery	42

tion with tetrahydrobiopterin can improve endothelial function. However, further studies are needed to reconcile the apparent discrepancy between elevation of endogenous tetrahydrobiopterin levels in atherosclerotic arteries and the beneficial effect of tetrahydrobiopterin supplementation on endothelial dysfunction in atherosclerosis. No study is currently available concerning the *in vivo* effect of chronic tetrahydrobiopterin supplementation on the progression of atherosclerosis. Shinozaki et al. (34) provided convincing evidence that chronic treatment with tetrahydrobiopterin prevents endothelial dysfunction in insulin-resistant rats. The beneficial effect of tetrahydrobiopterin appears to be due to the prevention of eNOS “uncoupling.” Tetrahydrobiopterin significantly reduced superoxide anion formation due to activation of eNOS. Furthermore, feeding with tetrahydrobiopterin prevented lipid peroxidation and activation of redox-sensitive transcription factors nuclear factor- κ B and activating protein-1. Interestingly, Shinozaki et al. (34) detected significant reduction of GTP cyclohydrolase I activity in aortas of insulin-resistant rats, suggesting that insulin may play an important role in control of tetrahydrobiopterin biosynthesis. Indeed, a study by Ishii et al. (17) demonstrated that insulin stimulates biosynthesis of tetrahydrobiopterin in cultured mouse brain microvascular cells. The exact mechanism underlying the stimulatory effect of insulin remains to be determined.

In attempts to employ tetrahydrobiopterin supplementation as a clinical strategy in the treatment of endothelial dysfunction, it is important to keep in mind that tetrahydrobiopterin does affect the biosynthesis of catecholamines. Tetrahydrobiopterin is a cofactor for aromatic amino acid hydroxylases [Michaelis constant (K_m) = 100–600 μ M]. Nitric oxide synthase(s) require about 1,000-fold less tetrahydrobiopterin for its activation than aromatic amino acid hydroxylases (K_m = 0.03–0.1 μ M). Thus elevation of tetrahydrobiopterin concentration may stimulate biosynthesis of catecholamines. Indeed, increased tetrahydrobiopterin synthesis induced by interleukin-2 cancer chemotherapy in humans is associated with an increase in serum levels of catecholamines (1). It is also important to keep in mind that tetrahydrobiopterin does not have selectivity for eNOS. Tetrahydrobiopterin stimulates the

enzymatic activity of nNOS and iNOS isoforms. This, in turn, may lead to excessive nitric oxide production and toxicity due to stimulation of iNOS enzyme activity in patients with severe infections, autoimmune disorders, and pathological angiogenesis (5). On the basis of current knowledge it is premature to recommend systemic administration of tetrahydrobiopterin in prevention and treatment of endothelial dysfunction. However, it is obvious that further studies of tetrahydrobiopterin vascular biology and pathology are needed.

IMPLICATIONS FOR THERAPEUTIC APPLICATION OF NOS GENE TRANSFER

During the past six years, numerous studies in animals demonstrated feasibility and potential utility of NOS gene transfer in the treatment of cardiovascular diseases (4). It is interesting that in intact arteries the beneficial effect of recombinant eNOS (or nNOS) was demonstrated without supplementation with tetrahydrobiopterin, suggesting that both in normal and diseased blood vessels, availability of tetrahydrobiopterin is not a limiting factor in the biosynthesis of nitric oxide. However, in most of these studies expression and function of recombinant eNOS and nNOS were studied during relatively short periods of time (up to 1 wk). Whether long-term elevation of eNOS (or nNOS) expression may require adjustments in tetrahydrobiopterin metabolism is unknown. Further studies are needed to determine whether in diseased blood vessels reduced availability of tetrahydrobiopterin may not only limit nitric oxide production, but create conditions for expression of “uncoupled eNOS”, leading to excessive production of superoxide anions, peroxynitrite, and oxidative injury.

Importance of optimal intracellular concentration of tetrahydrobiopterin for expression and function of recombinant iNOS has been reported. Supplementation with tetrahydrobiopterin increased nitric oxide production in cultured rat aortic smooth muscle cells transfected with iNOS (10). Interestingly, expression of recombinant iNOS in cerebral arteries is associated with a significant increase in superoxide anion production (Eguchi and Katusic, unpublished observations). Although the source and the mechanism of superoxide anions generation is unclear, it could be due to the “uncoupling” of iNOS. In endothelial and smooth muscle cells, induction of endogenous iNOS is associated with upregulation of GTP cyclohydrolase I, a rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin. In contrast, expression of recombinant iNOS in normal arteries is not coupled with increased expression and catalytic activity of GTP cyclohydrolase I. This can lead to a relative deficiency of tetrahydrobiopterin and “uncoupling” of iNOS. Thus supplementation with tetrahydrobiopterin may be required to avoid formation of superoxide anions and peroxynitrite by iNOS. Adverse effects of iNOS (as well as eNOS and nNOS) “uncoupling” in a gene therapy setting have not

been reported but certainly represent a potential source of vascular injury.

FUTURE DIRECTIONS

The function of tetrahydrobiopterin in NOS catalysis is still enigmatic. Further biochemical studies in this area are certainly warranted. With regard to vascular biology, very little is known about tetrahydrobiopterin metabolism in normal blood vessels. It will be of particular importance to improve the understanding of tetrahydrobiopterin metabolism in diseased arteries and veins. This may expand the knowledge needed for further refinement of therapeutic strategies directed toward prevention and treatment of endothelial dysfunction. In vitro findings demonstrating the ability of vitamin C to stimulate eNOS activity in cultured endothelial cells via chemical stabilization of tetrahydrobiopterin are the best illustration of the most recent progress in understanding the molecular mechanism underlying the protective effect of an important antioxidant. Genetic manipulation of GTP cyclohydrolase I (creation of knockout or transgenic mice) would have a major impact and would provide opportunities for cross-breeding with mice suffering from atherosclerosis, diabetes, or hypertension. These studies would certainly help to more precisely characterize the role of tetrahydrobiopterin in the pathogenesis of endothelial dysfunction. Availability of new-generation gene therapy vectors should improve long-term expression of recombinant NOS in arteries and veins. Experience obtained with these vectors will help to determine whether bioavailability of tetrahydrobiopterin may limit the ability of recombinant NOS(s) to restore nitric oxide production in dysfunctional endothelium.

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