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Review

Tetrahydrobiopterin and endothelial nitric oxide synthase activity

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1. Introduction

Nitric oxide (NO) plays a crucial role in the regulation of vascular tone and maintenance of vascular integrity [1–3]. Indeed, nitric oxide reduces vascular tone, inhibits leucocytes adhesion to the endothelium, platelet-vessel wall interaction, as well as vascular smooth muscle cell proliferation and migration. Accordingly, major risk factors for atherosclerotic vascular disease such as hypercholesterolemia, diabetes, hypertension and smoking, have been associated with impaired nitric oxide activity [4–11].

In vivo the activity of the L-arginine-NO pathway is a balance between the synthesis and breakdown of NO. Although, there are several reasons to believe that NO synthesis could be impaired [12–14], reduced NO activity could be caused by enhanced catabolism. Indeed, the in vivo half-life of NO is determined mainly by its reaction with oxyhemoglobin and superoxide anion [15]. Superoxide (O_2^-) may rapidly react with NO to produce peroxynitrite ($OONO^-$; [16]). This reaction is even faster than the one of O_2^- with superoxide dismutase to form hydrogen peroxide (H_2O_2) and molecular oxygen. High concentrations of $ONOO^-$ are very toxic, $ONNO^-$ can form peroxynitrous acid whose cleavage products are among the most reactive and damaging species in biological systems [17]. Taken together, these data indicate that catabolism of NO by its reaction with superoxide could be an important mechanism underlying endothelial dysfunction and oxidative vascular injury described in a number of vascular diseases [18,19]. It can be postulated that harmful concentrations of $ONOO^-$ can be achieved in a dysfunctional endothelium in which O_2^- generation is increased by cyclooxygenase, xanthine oxidase, and NADH oxidoreductase [20–22]. However, recent evidence indicates that decreased availability of tetrahydrobiopterin may be re-

sponsible for a dysfunction of nitric oxide synthase leading to a shift in the balance between the production of protective NO and deleterious oxygen-derived free radicals (Fig. 1). Tetrahydrobiopterin is known to be a cofactor of aromatic amino acid monooxygenases, which are regarded as key enzymes in the biosynthesis of several neurotransmitters, including catecholamines and serotonin [23]. It is indeed well established that inborn errors of tetrahydrobiopterin metabolism lead to cofactor deficiency, hyperphenylalaninemia, and neurological impairment [24]. In contrast, an important role of tetrahydrobiopterin in cardiovascular system has been recognized only recently. The first step of tetrahydrobiopterin biosynthesis involves activation of guanosine triphosphate (GTP)-cyclohydrolase I, which catalyzes the conversion of GTP to dihydroneopterin triphospahte [25]. Intracellular levels of tetrahydrobiopterin can also be increased by treating cells or animals with sepiapterin, which is converted to tetrahydrobiopterin via the so-called 'salvage pathway'. This review will briefly discuss the complex interaction between tetrahydrobiopterin and nitric oxide synthase and its role in the control of vascular tone.

2. Effects of tetrahydrobiopterin on NOS activity

NO is synthesized from L-arginine by nitric oxide synthase (NOS) through a five electron oxidation. Three distinct NOS isoforms have been identified by molecular cloning [26]. Two of them are expressed constitutively in neurones (neuronal) and vascular endothelial cells (endothelial) and are activated by increased intracellular calcium levels. The expression of a third isoform is induced, in a calcium-independent fashion, by various cytokines in macrophages and a number of other nucleated mammalian cells including hepatocytes and vascular smooth muscle

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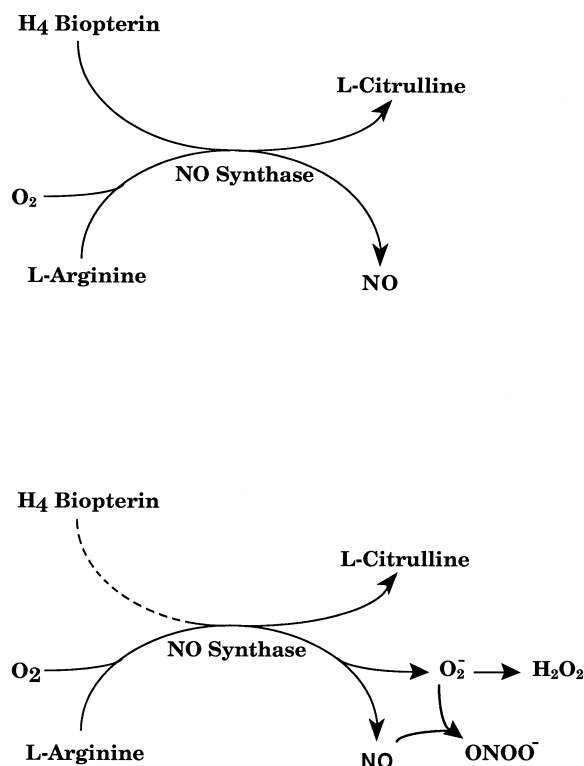*E-mail address:* 100771.1237@compuserve.com (T.F. Lüscher)

Fig. 1. Schematic representation of nitric oxide synthase (NO synthase) reaction leading to L-citrulline and nitric oxide (NO) from L-arginine and oxygen (O_2) (top). The activation of NO synthase at suboptimal levels (dashed line) of (6*R*)-5,6,7,8-tetrahydrobiopterin (H_4 Biopterin) generates superoxide anion (O_2^-) followed by the production of hydrogen peroxide (H_2O_2) and/or peroxynitrite ($ONOO^-$) from the rapid reaction of O_2^- and NO (bottom) (modified from ref. [38]).

(inducible). Purification and cloning of NOS isoforms have revealed that they are self-sufficient one-component cytochrome P-450s, which contain a prosthetic heme group catalyzing the reductive activation of molecular oxygen requisite for L-arginine oxidation, as well as tightly bound flavins shuttling NADPH-derived electrons to the heme (Fig. 2; [27]). Classical cytochrome P-450 hydroxylating systems would operate perfectly with these cofactors being

properly bound, but NO synthases do not. All three NOS isoforms additionally require tetrahydrobiopterin for catalytic activity. Tetrahydrobiopterin (H_4B) appears to mediate coupling of oxygen reduction to heme-catalyzed L-arginine oxidation to form NO and L-citrulline, but the molecular mechanism of this effect is still unknown. It could involve either an allosteric effect on the NOS protein or redox activity of H_4B, or both [25]. A close link between cellular H_4B availability and NO synthesis was recently demonstrated for a number of different cell types: murine endothelial cells, vascular smooth muscle cells, porcine and human endothelial cells, suggesting that the pathways for H_4B and NO synthesis are tightly coupled [25]. Indeed, in porcine and human vascular endothelial cells, inhibition of H_4B synthesis reduces production of NO in response to the calcium ionophore A23187 or bradykinin [28,29]. These studies provided evidence that in cultured endothelial cells, an optimal concentration of tetrahydrobiopterin is essential for agonist-induced, calcium-dependent production of nitric oxide.

Although, the precise role of this cofactor in regulation of NOS catalytic activity is still not completely understood [25], it has been postulated that H_4B plays an important role in whether the electron flow, within the enzyme, can be directed to L-arginine. Indeed, several biochemical studies demonstrated that activation of purified constitutive NOS in the presence of suboptimal levels of tetrahydrobiopterin results in uncoupling of oxygen reduction and arginine oxidation, thereby generating superoxide anions and subsequently hydrogen peroxide [30–32]. In agreement with these results, we recently demonstrated that in isolated canine coronary arteries depleted of tetrahydrobiopterin endothelial nitric oxide synthase may become a source of oxygen-free radicals [33]. Ever since, growing evidence indicates that, under certain pathological conditions, decreased tetrahydrobiopterin availability may be responsible for dysfunction of endothelial NOS.

Very intriguing are recent findings showing that endothelial cells that were incubated with LDL released superoxide, which could be inhibited by the NOS inhibitor
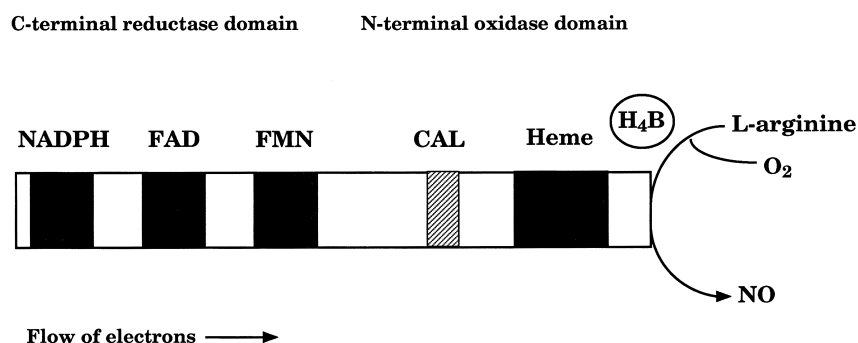

Fig. 2. NO is produced by NOS, which incorporates molecular oxygen into the substrate L-arginine. The NOS itself has binding sites for tetrahydrobiopterin (H_4B), L-arginine and heme. Electrons donated by reduced nicotinamide-adenine dinucleotide phosphate (NADPH) are shuttled through the reduced flavins, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) toward the oxidase domain. The built-in electron transport system is used to oxidize L-arginine to NO and L-citrulline. This reaction is dependent on the presence of H_4B.

L-NAME [34]. These data suggest that NOS itself can be an important source for endothelial superoxide production in hypercholesterolemia. Indeed, enhanced oxidative degradation of NO is a major determinant of impaired NO activity in hypercholesterolemia [35–37]. Deficiency of tetrahydrobiopterin causes both impaired NO activity and increased oxygen radical formation [33,38]. In this regard, we further demonstrated that infusion of tetrahydrobiopterin into the brachial artery of patients with hypercholesterolemia restores endothelial dysfunction by increasing production of NO [39]. Therefore, increased breakdown of nitric oxide could be explained from a decreased availability of tetrahydrobiopterin. Cofactor supplementation may restore NO activity by decreasing oxygen radical formation. Tetrahydrobiopterin can also improve abnormal endothelium-dependent coronary vasomotion in response to acetylcholine in patients with coronary artery disease [40]. Furthermore, administration of tetrahydrobiopterin is capable of restoring endothelium-dependent vasodilation in experimental diabetes [41], smoking [42,43], and reperfu-

sion injury [44]. Such effect of exogenous tetrahydrobiopterin is consistent with the concept of an altered tetrahydrobiopterin-NOS interaction which may lead to the above-mentioned dysfunctional activity of the enzyme.

Interestingly enough, an impaired synthesis of H_4B occurs in adrenal cortex of spontaneously hypertensive rats (SHR; [45]). This metabolic dysfunction was detected in prehypertensive animals suggesting that it may contribute to the development of hypertension and/or its complications. We reported [38] that in isolated aortas from prehypertensive SHR, H_4B supplementation diminished the NOS-dependent generation of superoxide and its dismutase product hydrogen peroxide, while it increased the net production of NO (Fig. 3). Although, the levels of H_4B from SHR were not different when compared to Wistar-Kyoto (WKY) aortas, NOS activity in response to exogenous H_4B was significantly higher in the latter. These results suggest that an increased requirement for H_4B may trigger an uncoupling of the oxidative and reductive domain of the enzyme resulting in dysfunctional
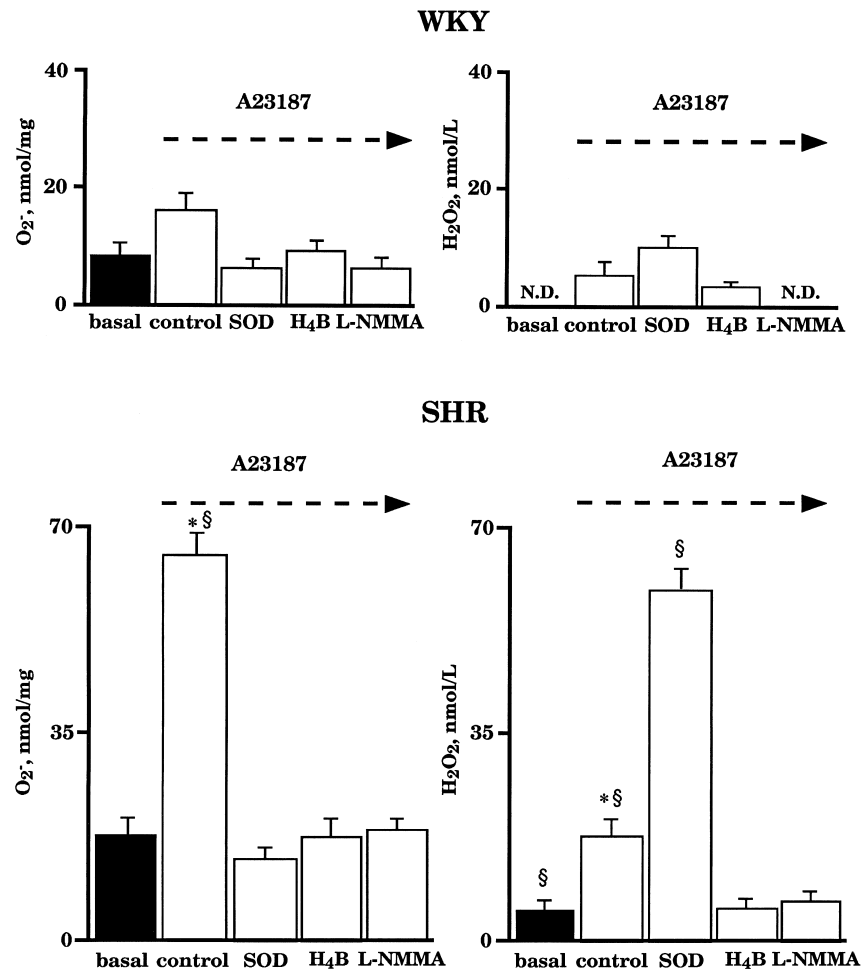

Fig. 3. Bar graphs showing the basal and calcium ionophore A23187-stimulated concentration of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in aortic tissue from 4-week old Wistar-Kyoto rats (WKY, top) and spontaneously hypertensive rats (SHR, bottom). Note that after tetrahydrobiopterin (H_4B) supplementation and in the presence of NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) the A23187-induced concentrations of O_2^- and its dismutase product H_2O_2 were significantly reduced only in SHR (reprinted from ref. [38]).

NOS activity. Whether oxygen free radicals formed via NOS plays a role in the development of hypertension remains to be determined.

3. Conclusions

All together these observations strongly support the concept of a dysfunctional NOS as a new source of reactive oxygen metabolites. This NOS-catalyzed formation of O_2^- and its subsequent transformation into HONOO cleavage products, or its dismutation into H_2O_2 and Fenton reaction product OH, may play a pivotal role in the endothelial dysfunction and oxidative vascular injury described in a number of vascular diseases. Therefore, reduced availability of H_4B may represent an important mechanism underlying conditions associated with impaired NO activity and accelerated atherosclerosis. Although the background for such a deficiency is not clear, these findings warrant further exploration for a better understanding of signal transduction pathways sustaining the formation of H_4B in the endothelium. The present knowledge not only underscore the relevance of H_4B as crucial cofactor for NO synthesis but also may initiate research into new therapeutic approaches to reduce cardiovascular risk.

Acknowledgements

References

[1] Palmer RMJ, Ferrige AG, Moncada S. Nitric release accounts for the biological activity of endothelial-derived relaxing factor. Nature 1987;327:534–536.

[2] Furchgott R, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;299:373–376.

[3] Lüscher TF, Noll G. The pathogenesis of cardiovascular disease: role of the endothelium as target and mediator. Atherosclerosis 1995;188(Suppl.):S81–S90.

[4] Creager M, Cooke JP, Mendelsohn ME et al. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic patients. J Clin Invest 1990;86:228–234.

[5] Egashira K, Inou T, Hirooka Y et al. Impaired coronary blood flow response to acetylcholine in patients with coronary risk factors and proximal atherosclerotic lesions. J Clin Invest 1993;91:29–37.

[6] Egashira K, Inou T, Hirooka Y et al. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. Circulation 1993;88:77–81.

[7] Stroes ESG, Koomans HA, de Bruin TWA, Rabelink TJ. Vascular function in the forearm of hypercholseterolemic patients off and on lipid-lowering medication. Lancet 1995;346:467–471.

[8] Panza JA, Garcia CE, Kilcoyne CM, Quyyumi AA, Cannon RO. Impaired endothelium-dependent vasodilation in essential hypertension. Circulation 1995;91:1732–1738.

[9] Johnstone MT, Creager SJ, Scales KM et al. Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes. Circulation 1993;88:2510–2516.

[10] Ting HT, Timimi FK, Boles KS et al. Vitamins C improves endothelium-dependent vasodilation in patients with non insulin-dependent diabetes mellitus. J Clin Invest 1996;97:22–28.

[11] Heitzer TS, Yla-Herttuala S, Luoma J et al. Cigarette smoking potentiates endothelial dysfunction of forearm resistance vessels in patients with hypercholesterolemia. Circulation 1996;93:1346–1353.

[12] Freeman JE, Kuo WY, Drenger B et al. Analysis of lysophosphatidylcholine-induced endothelial dysfunction. J Cardiovasc Pharmacol 1996;28:345–352.

[13] Bode-Boger SM, Boger RH, Kienke S, Junker W, Frolich JC. Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. Biochem Biophys Res Commun 1996;219:598–603.

[14] Cooke JP, Tsao PS. Arginine: a new therapy for atherosclerosis? Circulation 1997;95:311–312.

[15] Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad and the ugly. Am J Physiol 1996;271:C1424–C1437.

[16] Kobayashi K, Miki M. A direct demonstration of reaction of nitric oxide with superoxide anion by the use of pulse radiolysis. In: Asada K, Yoshikawa T, editors, Frontiers of reactive oxygen species in biology & medicine, Elsevier Science B.V, Amsterdam, 1994, pp. 223–224.

[17] Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. In: Packer L, editor, Part C, Oxygen radicals in biological systems, Methods of enzymology, Vol. 233, Academic Press Inc, San Diego, CA, 1994, pp. 229–240.

[18] Pryor WA. Free radicals and lipid peroxidation: what they are and how they got that way. In: Frei B, editor, Natural antioxidants in human health and disease, Academic Press, Boston, 1994, pp. 1–24.

[19] Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 1994;344:721–724.

[20] Cosentino F, Sill JC, Katusic ZS. Role of superoxide anions in mediation of endothelium-dependent contractions. Hypertension 1993;23:229–235.

[21] Kontos HA. Oxygen radicals in cerebral vascular injury. Circ Res 1985;57:508–516.

[22] Mohazzab KM, Kaminski PM, Wolin MS. NADH oxidoreductase is a major source of superoxide anion in bovine artery endothelium. Am J Physiol 1994;266:H2568–H2572.

[23] Kaufman S. New tetrahydrobiopterin-dependent systems. Annu Rev Nutr 1993;13:261–286.

[24] Blau N, Thony B, Heizmann CW, Dhont JL. Tetrahydrobiopterin deficiency: from phenotype to genotype. Pteridines 1993;4:1–10.

[25] Mayer B, Werner ER. In search of a function for tetrahydrobiopterin in the biosynthesis of nitric oxide. Naunyn-Schmied Arch Pharmacol 1995;351:453–463.

[26] Knowles RG, Moncada S. Mammalian nitric oxide synthase. Biochem J 1994;298:249–258.

[27] Bredt DS, Hwang PM, Glatt CE et al. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 1991;351:714–718.

[28] Schmidt K, Werner ER, Mayer B, Wachter H, Kukowetz WR. tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. Biochem J 1992;281:297–300.

[29] Werner-Felmayer G, Werner EG, Fuchs D et al. Pteridine biosynthseis in human endothelial cells: impact on nitric oxide-mediated formation of cGMP. J Biol Chem 1993;268:1842–1846.

[30] Stuher DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF. Purification and characterization of the cytokine-induced macrophage nitric

oxide synthase: an FAD and FMN-containing flavoprotein. Proc Natl Acad Sci USA 1991;88:7773–7777.

[31] Heinzel B, John M, Klatt P, Bohme E, Mayer B. Ca^{2+}/calmodulin dependent formation of hydrogen peroxide by brain nitric oxide synthase. Biochem J 1992;281:627–630.

[32] Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. Generation of superoxide by purified brain nitric oxide synthase. J Biol Chem 1992;267:24173–24176.

[33] Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. Circulation 1995;91:139–144.

[34] Pritchard KA, Groszek L, Smalley DM et al. Native low density lipoprotein increaseas endothelial cell nitric oxide synthase generation of superoxide anion. Circ Res 1995;77:510–518.

[35] Harrison DG, Ohara Y. Physiologic consequences of increased vascular oxidant stress in hypercholesterolemia and atherosclerosis: implications for impaired vasomotion. Am J Cardiol 1995;75:75B–81B.

[36] Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest 1993;91:2546–2551.

[37] Ting HH, Timimi FK, Haley EA et al. Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessel of humans with hypercholesterolemia. Circulation 1997;95:2617–2622.

[38] Cosentino F, Patton S, d'Uscio LV et al. Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. J Clin Invest 1998;101:1530–1537.

[39] Stroes E, Kastelein J, Cosentino F et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 1997;99:41–46.

[40] Maier W, Cosentino F, Seiler C et al. Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. Eur Heart J 1997;18:15.

[41] Pieper GM. Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. J Cardiovasc Pharmacol 1997;29:8–15.

[42] Higman DJ, Strachan AMJ, Buttery L et al. Smoking impairs the activity of endothelial nitric oxide synthase in saphenous vein. Artherioscl Thromb Vasc Biol 1996;16:546–552.

[43] Heitzer T, Munzel T. The cofactor of the nitric oxide synthase tetrahydrobiopterin improves endothelial dysfunction in smokers. Circulation 1998;98(17):I–735.

[44] Tiefenbacher CP, Chilian WM, Mitchell M, DeFily DV. Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. Circulation 1996;94:1423–1429.

[45] Abou-Donia MM, Daniels JA, Nichol CA, Viveros HL. Regulation of adrenocortical guanosine triphosphate cyclohydrolase and tetrahydrobiopterin in normal and spontaneously hypertensive rats. In: Blair JA, editor, Chemistry and biology of pteridines, Walter de Gruyter and Co, New York, 1983, pp. 783–787.