Pteridines
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Tetrahydrobiopterin in the vascular system

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Introduction

Since the initial description of a pteridine (Fig. 1) in Nature 112 years ago,(1) numerous studies contributed to the current understanding of these small molecules as ubiquitous and essential compounds required for

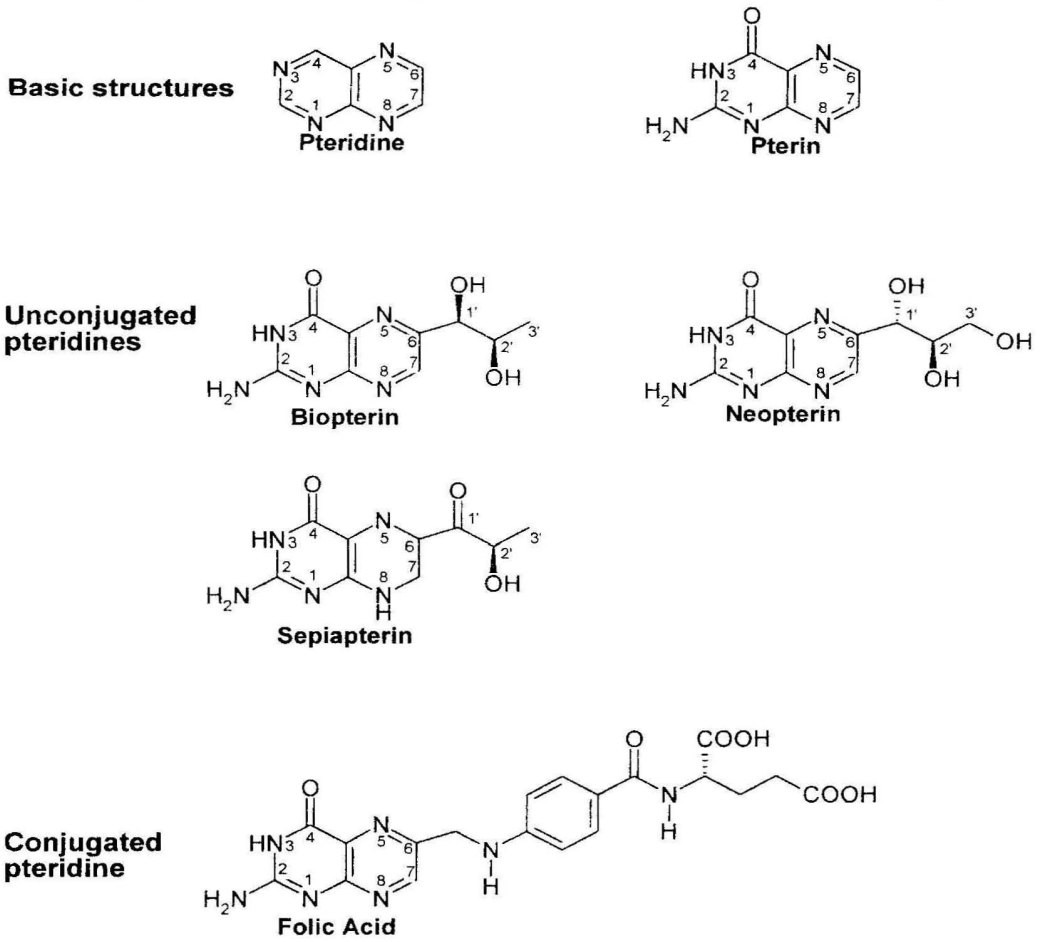

Figure 1. Chemical structures. "Pteridine" denotes the pyrazino-2,3-d-pyrimidine ring system underlying these molecules. The majority of the naturally occurring unconjugated pteridines, e.g. biopterin, neopterin, or sepiapterin, are derivatives of the 2-amino-4-oxo-pteridine, and this bicyclic basic structure has been termed "pterin". Note that sepiapterin is not synthesized in mammals including man. Besides unconjugated pteridines, also conjugated pteridines are widely distributed in nature. Certainly the most important are folates, a group of heterocyclic compounds based on the 4-[(pteridin-6-ylmethyl)amino]benzoic acid skeleton conjugated with one or more L-glutamate units.

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abundant cellular functions in plants, bacteria, non-mammals, and mammals.

In man, tetrahydrobiopterin (BH_4) is the predominant and most important pteridine. Research on BH_4 first focused on the recognized cofactor function during aromatic amino acid hydroxylation and diseases associated with inborn errors of BH_4 metabolism.2 However, a century after the publication of Hopkins' short notice, it was apparent that BH_4 has additional metabolic roles, and major investigators' interest turned to the relationship between these compounds and the immune system.(2) A link was made with the observation that BH_4 is also essentially required for synthesis of nitric oxide (NO),(3-6) a lipid- and water-soluble gas believed to be central for host defense.(7;8) Concomitantly, a pivotal role of NO in the control of vascular tone became established as well,(9-11) and soon data indicated that BH_4 might play a critical role in the vascular system. This review will focus on that role of BH_4 with emphasis on human experimental and clinical studies and will discuss the potential of BH_4 as novel treatment modality for vascular disorders. Other facets of BH_4 biology have been covered in recent reviews.(2;12-23) At the turn of the millenium, however, a number of important questions remain unanswered certainly stimulating future work in this field.

Materials and Methods

BIOSYNTHESIS OF BH_4 AND ITS REGULATION

The molecular basis of BH_4 biosynthesis and regeneration has been characterized in detail. Today, stuctures and kinetics of involved enzymes are known, and corresponding human genes have been localized and cloned; for recent review see.(13)

BH_4 biosynthesis and regeneration (Fig. 2)

De novo biosynthesis of BH_4. De novo biosynthesis requires three main enzymatic steps. In a first step, GTP is cleaved by GTP cyclohydrolase I (GTPCH) to yield 7,8-dihydroneopterin triphosphate in a complex catalytic mechanism resulting in several intermediates. 7,8-dihydroneopterin triphosphate is then converted to 6-pyruvoyl-5,6,7,8-tetrahydropterin (PTP) in a Zn^{2+}- and Mg^{2+}-dependent reaction by 6-pyruvoyl tetrahy-

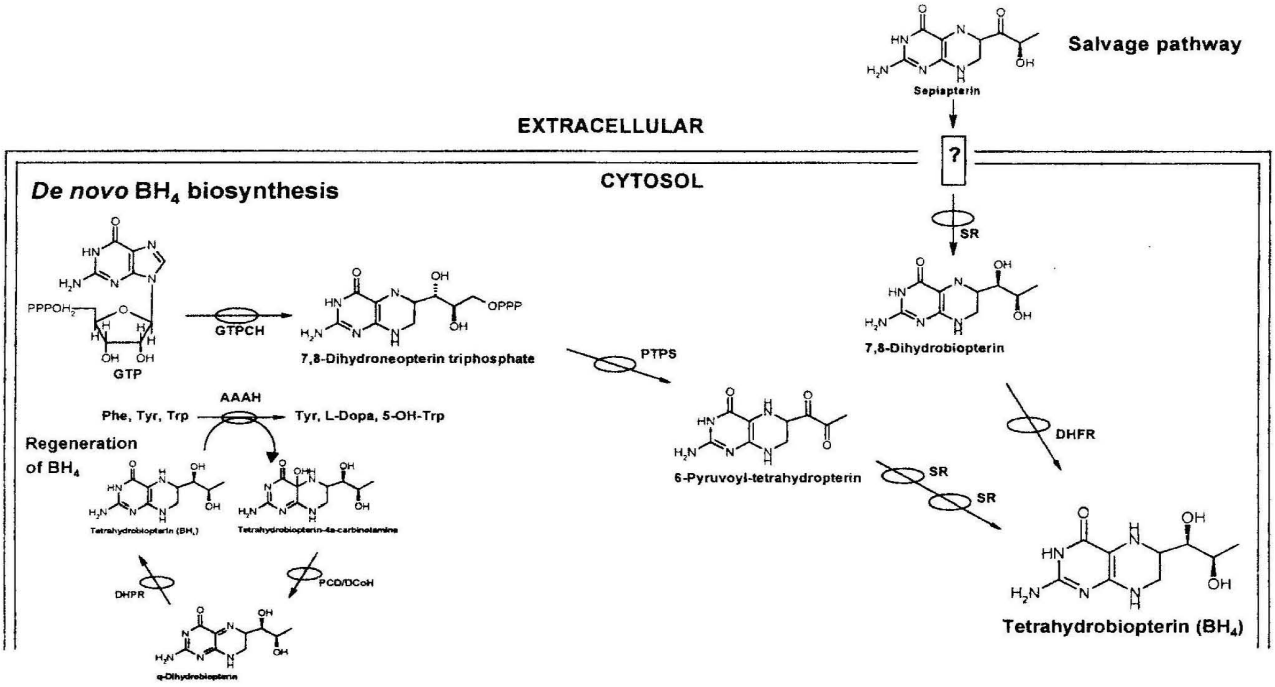

Figure 2. BH_4 biosynthesis, salvage pathway, and regeneration. See text for details. De novo biosynthesis starts from GTP. Sepiapterin is not synthesized in mammalian cells, but is taken up by an yet unknown mechanism. BH4 oxidized during aromatic amino acid hydroxylation is recycled by a separate enzyme system. Abbreviations: AAAH, aromatic amino acid hydroxylase; DHFR, dihydrofolate reductase; DHPR, dihydropteridine reductase; GTPCH, GTP cyclohydrolase I; PCD/DCoH, pterin-4a-carbinolamine dehydratase; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase.

dropterin synthase (PTPS) without the need for external reducing agents. Finally, sepiapterin reductase (SR) catalyzes the reduction of the two side-chain keto groups of PTP to BH_4 in a two-step reaction each requiring NADPH as a redox cofactor.

Regeneration of BH_4. During the catalytic process of amino acid hydroxylases, BH_4 is oxidized to BH_4-4a-carbinolamine.This compound is further dehydrated to quinoid dihydrobiopterin by pterin-4a-carbinolamine dehydratase (PCD/DCoH). Finally, the dimeric dihydropteridine reductase (DHPR) reduces quinoid dihydrobiopterin back to BH_4 in a NADH-dependent reaction.

Salvage pathway. Via the pterin salvage pathway, preexisting 7,8-BH_2 and sepiapterin, a pteridine not naturally occuring in mammals, can be converted to BH_4. Sepiapterin is converted to 7,8-BH_2 by SR. In the final step, BH_4 is formed in a NADPH-dependent reaction by dihydrofolate reductase (DHFR).

Regulation of enzyme activity involved in BH_4 biosynthesis and regeneration

GTPCH is commonly thought to be the rate-limiting enzyme in de novo BH_4 biosynthesis. Its activity is regulated at the transcriptional and post-translational level as well as via GTPCH feedback regulatory protein (GFRP) which modulates enzymatic activity by inducing an conformational change of GTPCH. Regulation at the transcriptional level by pro- and anti-inflammatory cytokines or endotoxin may be remarkable and the predominant mechanism for modulation of enzymatic activity. Compared to rodent cells, human cells have much lower PTPS activity. Therefore, 7,8-dihydroneopterin triphosphate is not efficiently converted into BH_4 but is partially dephosphorylated to 7,8-dihydroneopterin by cellular phosphatases and further oxidized to neopterin. The rate-limiting effect of PTPS in human cells is reflected by accumulation of neopterin following immunostimulatory induction of GTPCH activity. Very recently, a cytokine-dependent regulation of PTPS mRNA abundance and specific activity in human vascular endothelial cells that prevents neopterin accumulation has been shown. In contrast, no significant modulation of enzymatic activity of SR has been found in vivo so far, although in vitro studies showed modification of kinetic properties following phosphorylation. Whether the enzymatic activity of PCD/DCoH, DHPR, or DHFR is regulated to a clinically important degree is unknown at present.(13;17;21)

FUNCTIONS OF BH_4

BH_4 as substrate of aromatic amino acid hydroxylases

Phenylalanine hydroxylase, tyrosine 3-hydroxylase, and tryptophan 5-hydroxylase constitute a family of enzymes which are central to normal physiology because they modulate the production of neurotransmitters and hormones such as dopamine, norepinephrine, epinephrine, serotonin, and melatonin.(15;24;25) These homotetrameric enzymes have a strict requirement for pteridines, oxygen, and iron. BH_4 has been identified as the naturally occurring cofactor, but a variety of substitutes at different positions of the pterin ring are tolerated by at least one of the monooxygenases. The pyrimidine, but not the complete pyrazine ring is required for enzymatic activity. During catalysis, one atom of oxygen from molecular oxygen is incorporated into the substrate and the other atom reduced to water. BH_4, which acts as substrate rather than tightly bound cofactor, supplies both electrons required for the reduction of the second atom of water. One of the oxidation products of the catalytic reaction by all three hydroxylases is BH_4-4a-carbinolamine, which is subsequently regenerated by the enzymes PCD/DCoH and DHPR.

Phenylalanine hydroxylase is allosterically regulated by phenylalanine and BH_4. Whereas phenylalanine is a positive allosteric effector that converts the inactive enzyme to a catalytically active form, BH_4 is a negative effector that competes with phenylalanine to form a dead-end complex with the inactive enzyme.(26;27) A further regulation of phenylalanine hydroxylase activity is achieved by phosphorylation of a serine residue resulting in a shift of the balance between the active and inactive form of the enzyme towards the active form. BH_4 has been recognized to inhibit this phosphorylation.(15) All amino acid hydroxylases are inhibited by catecholamines, but only the inhibition of human tyrosine hydroxylase is competitive with respect to BH_4.

BH_4 as cofactor for NO synthase (NOS)

Catalysis by NOS (EC 1.14.13.39) is complex and molecular details of the reaction mechanism remain elusive; for recent comprehensive reviews see.28-34 The NOS family comprises at least three distinct homodimeric enzymes: neuronal (nNOS, NOS1), inducible (iNOS, NOS2) and endothelial (eNOS, NOS3). They have considerable similarity in catalytic function and share about 50% homology in their amino acid sequences. NOSs exhibit spectroscopic and catalytic properties strongly reminiscent of those of cytochrome P450 reductase and are members of the heme-thiolate family of proteins that also includes P450.

NOS catalyzes the five electron oxidation of one of

the chemically equivalent guanidino-nitrogens of L-arginine in a two-step process to finally yield L-citrulline and NO via N^{ω}-hydroxy-L-arginine as tightly-bound intermediate. Due to the labile nature of NO, direct evidence that purified NOS directly synthesize NO has only recently been provided by electron paramagnetic resonance spectroscopy.(35)

NOS isoforms differ primarily with respect to subcellular localization, expressional regulation, post-translational modifications, and activity modulation. nNOS contains a PDZ binding motif (found in a number of proteins implicated in ion-channel and receptor clustering, and the linking of receptors to effector enzymes. PDZ domains are protein-recognition modules) at the amino terminus that is involved in subcellular targeting of the protein, and eNOS is distinguished by the presence of myristoylation, palmitoylation, farnesylation, acetylation, and phosphorylation sites at the amino terminus that serve a similar function. Depalmitoylation of eNOS promotes the dissociation of the enzyme from its proximity to activating molecules localized in the membrane and may serve as a feedback mechansim leading to eNOS deactivation. eNOS and nNOS are considered constitutive and participate in signal cascades by synthesizing NO depending upon elevation of intracellular calcium. Main regulation of enzymatic activity occurs post-translationally. iNOS is expressed in various cell types in response to certain stimuli, such as inflammatory cytokines or bacterial cell products, and its regulation occurs mostly at the level of transcription. With tightly bound calmodulin, iNOS is fully active at basal calcium levels.

In all NOS isoforms, BH_4, heme (iron protoporphyrin IX), flavin mononucleotide (FMN), and flavin adenin dinucleotide (FAD) are required as cofactors, molecular oxygen is used as cosubstrate, and NADPH donates the reducing equivalents necessary for enzymatic activity. Each monomer has two domains: a N-terminal oxygenase domain (containing BH_4 and heme) and a C-terminal reductase domain (containing FAD and FMN) linked by a calmodulin-binding consensus sequence. All NOS isoforms dimerize through the heme domain. An unified model has been proposed to explain the enzymatic mechanisms of NOSs. Critical to NOS catalysis is the transfer of electrons from NADPH to heme. Binding of calmodulin serves to position the two domains and promotes the flow of NADPH-derived electrons from the flavins to the heme, which is thought to activate oxygen and to initiate NO synthesis. Maximum rate of NO synthesis by eNOS is severalfold slower than that of nNOS or iNOS, primarily due to low intrinsic reductase activity that limits both the delivery of electrons to the heme and overall activity of the enzyme.

BH_4 is absolutely required in the catalytic reaction of NOS,(3-6) however, the exact role in the enzymatic process is still incompletely understood. It binds by hydrogen bridges with a heme propionate in a pocket situated between monomers where it interacts with residues from each subunit, and may thereby stabilize the dimeric form of the enzyme. In fact, BH_4 together with the substrate L-arginine promotes the dimerization of iNOS and stabilizes the nNOS dimer; however, it does not seem to be required for the dimerization of eNOS. Modulation of NOS activity by BH_4 is exclusively mediated through interactions with the heme domain. Both the pteridine cofactor and L-arginine interact with the same heme propionate which explains the interplay between BH_4 and substrate binding. Indeed, allosteric effects of BH_4 on NOS have long been described. BH_4 triggers the conversion of the inactive low-spin to the active high-spin conformation of the enzyme, and BH_4 interacts with L-arginine by positive cooperativity. An unusual aspect of BH_4 binding is that two identical but highly anticooperative binding sites for BH_4 have been found for nNOS and iNOS. With binding of a first BH_4 to the NOS dimer, one L-arginine is tightly bound, and this greatly weakens the affinity of the second BH_4 and L-arginine binding sites in the NOS dimer for both, BH_4 and L-arginine, respectively. It is therefore possible that NOS could contain only one BH_4 per dimer in some biological settings. For human nNOS, dual allosteric interactions between BH_4 binding site and L-arginine-binding site have been confirmed. BH_4 binding not only increases enzyme activity but also decreases the apparent Km for L-arginine, and, conversely, L-arginine activates human nNOS by allosterically modulating/increasing the affinity for binding of BH_4. The 1,2-dihydroxypropyl side chain at the 6-position has been suggested to promote cooperativity of BH_4.(36)

Recent data indicate a redox-active role of BH_4 in the NOS reaction. Experiments with BH_4-free NOS indirectly implicate the reduced pteridine cofactor in the first step of the reaction. Together with the newly recognized participation of nonheme ferrous iron in the reaction, the current information raise the possibility that the pteridine may act in this step in an analogous fashion to that characterized for the amino acid hydroxylases. Furthermore, it has been established that BH_4 promotes the transition of the heme group from low to high spin-state thereby facilitating electron transfer from the flavoprotein domain resulting in the reduction of heme-iron from ferric to the ferrous form, which is required for the conversion of L-arginine to N^{ω}-hydroxy-L-arginine. Critical to all mechanistic

considerations is the formation of the ferrous-dioxygen NOS complex during the second step of NOS catalysis. BH_4 decreases the lifetime of this complex (as shown for nNOS) and may therefore influence the course of the reaction by affecting electron transfer. Recent low temperature experiments suggested that BH_4 may donate an electron required for activation of the ferrous dioxygen complex thereby generating a pteridine radical, which is rapidly reduced intraenzymically thereafter as part of the NOS catalytic cycle.

Enzyme-generated NO can bind to the NOS heme and influence subsequent catalysis in a reversible manner as the NO-bound form is inactive ("NO feedback inhibition"). Both iNOS and nNOS intrinsically form heme-NO complexes during NO synthesis, and a majority of enzyme may partition into the inactive form due to insufficiently slow dissociation of NO from NOS. BH_4 has been shown to attenuate NO feedback inhibition thereby stimulating enzymatic activity. Possible mechanisms may include removal of NO by reaction with superoxide formed in the course of BH_4 autoxidation, and destabilization of the heme-NO complex. In contrast, little or no NO complex is formed during NO generation from eNOS, most probably due to slow electron transfer between flavins and heme resulting in a low NO concentration. These findings indicate that NO feedback inhibition has a minimal role in eNOS catalysis.(37)

Recent data suggest that intracellular BH_4 availability may regulate iNOS mRNA and protein expression. This effect appears to be mediated, at least in part, by an increase in iNOS mRNA stability.(38;39)

An important aspect of NOS catalysis is the formation of oxygen-derived free radicals as a general feature of all NOSs which has been studied over the recent years in detail by use of electron paramagnetic resonance spin-trapping experiments with purified NOS. Evidence indicates that BH_4 plays a critical role not only in increasing the rate of NO generation but also in controlling the formation of superoxide and hydrogen peroxide by NOS.

Besides synthesizing NO, nNOS can catalyze superoxide formation at low levels of L-arginine in a calcium/calmodulin-dependent process.(35;40-42) In vitro studies using nNOS transfected cells showed that NO production declined with L-arginine depletion whereas the production of superoxide increased, and this switch of NOS from the production of NO to superoxide crucially depended on intracellular levels of L-arginine.(43) The source of superoxide remains unclear. Some investigators suggested the formation of superoxide at the oxygenase domain of nNOS, whereas others implicated the reductase domain of nNOS, or both.(44)

L-arginine controls the generation of superoxide by decreasing the rate of NADPH consumption.(44) In assays with purified nNOS protein, superoxide may also be generated by the reductive reaction of high concentrations of FAD with NADPH.(35) In addition, in the presence of a redox-cycling compound such as FMN, nNOS increases the generation of superoxide in a calcium/calmodulin-independent manner.(44)

Occupation of the L-arginine binding site may stabilize the ferric superoxide complex,(45) and the prevention of dissociation of this complex is proposed to decrease the generation of superoxide both from the oxygenase and reductase domains of nNOS.(44)

Pteridine-free purified nNOS generates superoxide from the reductase and the oxygenase domain by a calcium/calmodulin-dependent mechanism. Generation of superoxide by this pathway in tightly controlled by BH_4, possibly by promoting the formation of heme-peroxo species. In the presence of both L-arginine and BH_4, the formation of the oxoferryl will occur thereby facilitating the oxidation of L-arginine to generate N?-hydroxy-L-arginine.44 BH_4 and L-arginine also stabilize dimeric nNOS during catalysis and markedly inhibit monomerization, thereby reducing enzyme inactivation induced by the generation of reactive oxygen species. It has been proposed that BH_4 may directly scavenge excessively generated superoxide and subsequently degrade to yet unknown products in situations when L-arginine levels are limiting.(36)

Hydrogen peroxide may be formed by nNOS by two different mechanisms depending on the availability of BH_4. At low BH_4 concentrations, nNOS will generate only superoxide that, by dismutation, will produce hydrogen peroxide and oxygen. In the presence of BH_4, however, no superoxide will be formed, and the enzyme will generate hydrogen peroxide by a mechanism involving a two-electron reduction of oxygen.(44) BH_4 decreases the generation of hydrogen peroxide by nNOS while increasing L-citrulline formation.(41;46)

Superoxide and NO synthesis can also occur simultaneously within iNOS in experiments with purified protein,(47) and in L-arginine depleted macrophages iNOS has been shown to generate peroxynitrite,(48) a powerful oxidant with a relatively long half-life that is formed at a diffusion-limited rate from NO and superoxide.(49) Superoxide generation mainly occurs at the flavin-binding sites of the reductase domain.(47) In experiments using BH_4-free iNOS expressed in E. coli, BH_4 shifted iNOS from a superoxide producing to a peroxynitrite producing enzyme in the absence of superoxide dismutase (SOD, EC 1.15.1.1); only the addition of SOD resulted in a detectable NO signal.(50)

iNOS and nNOS exhibit very different L-arginine-dependent inhibition of superoxide generation. Inhibition of superoxide generation from iNOS was only seen at high L-arginine concentrations, whereas low L-arginine levels completely block superoxide formation from nNOS.(41;47) The reason for this difference is not yet clear. However, it has been concluded that because of relative high cytosolic levels of L-arginine, superoxide generation would rarely occur with nNOS, whereas the simultaneous NO and superoxide generation from iNOS may be beneficial since these two free radicals will interact to form the more potent toxic oxidant peroxynitrite which will prevent feedback inhibition of iNOS and may enhance killing activity by iNOS.(47)

Superoxide can be generated by eNOS in a calcium/calmodulin-dependent manner from the dissociation of a transient heme ferrous-dioxygen complex in the oxygenase domain of eNOS. BH_4 dose-dependently decreases superoxide levels both by coupling L-arginine oxidation to NADPH consumption and by direct scavenging properties. In contrast to the effect of BH_4, L-arginine does not control superoxide generation and does not interfere with the release of superoxide from heme iron. Therefore, eNOS mediated superoxide generation is triggered and controlled by decreased availability of BH_4 rather than L-arginine leading to a switch of eNOS from NO to superoxide production in states of BH_4 depletion.(51;52) Most interestingly, preliminary data indicate that the ratio of BH_4 to 7,8-BH2 may control superoxide generation as BH_4-oxidized derivatives compete with BH_4 for the BH_4-binding site at the oxygenase domain of eNOS resulting in uncoupling and increased superoxide production.(53)

NO interacts with various intracellular molecular sites within both the generating and target cell, a distinct NO receptor does obviously not exist. The biology of NO signaling has recently been reviewed in detail.(54) Direct effects of NO include interactions with transition metals leading to formation of stable nitrosyl complexes via covalent reactions between NO and metal ions, redox reactions between NO and metal ions, and NO binding to iron-sulfur clusters in proteins. Overall, the interaction of NO with the heme component of soluble guanylate cyclase with subsequent stimulation of enzymatic conversion of GTP to cyclic guanosine 3',5'-monophosphate (cGMP) constitutes the major pathway of NO signaling. Other direct effects of NO include reactions with free radicals. In states of sustained and enhanced NO flux, reactive nitrogen species are formed, and indirect effects of NO are consequences of S-nitrosation reactions. Most, if not all of these indirect effects of NO are dependent on reactions with oxygen and superoxide, giving rise to dinitrogen trioxide and peroxynitrite.(54) Some of these direct and indirect effects of NO in the vasculature are modulated by BH_4 and are discussed below.

BH_4 as cofactor for other enzymes

BH_4 is involved in the reactions of at least three other enzymes. The reduced pteridine is required by the glyceryl-ether mono-oxygenase (EC 1.14.16.5) for hydroxylation of the a-carbon atom of the lipid carbon chain of glyceryl ether to form a-hydroxyalkyl glycerol.(55) Furthermore, the dopamine-b-hydroxylase (EC 1.14.17.1) in the pathway from dopamine to noradrenalin also depends on BH_4.(56) Finally, BH_4 can directly control tyrosinase (EC 1.14.18.1) activity, the key enzyme in melanin biosynthesis in melanocytes, by uncompetitive inhibition via a specific binding domain on the enzyme.(57) BH_4 has also been suggested to participate as physiological coenzyme in the catalysis of indoleamine 2,3-dioxygenase (EC 1.13.11.42),(58;59) however, these findings have been questioned by others.(2)

Other functions of BH_4

As mentioned above, the first cellular function of BH_4 recognized was the promotion of growth for the protozoon *Crithidia fasciculata*;(60) microbiological assays using the nutritional requirement of this organism for the L-erythro configuration of biopterin have been developed for quantification of this pterin in diferent body fluids and tissues and utilized for some time; however, they needed a period of several days to obtain accurate results, and they were finally replaced by novel and even more sensitive detection techniques, e.g. radioimmunoassays, gas chromatography mass spectrometry, or high-performance liquid chromatography.(61;62)

In addition to its cofactor role for NOSs and aromatic amino acid hydroxylases, other functions of BH_4 in the nervous system have been suggested. These include a role as a neurotransmitter-releasing factor, first demonstrated for dopamine in the rat striatum (.63) This effect may be mediated by activation of neuronal calcium channels via cAMP-protein kinase A pathway independent of cofactor activity, for recent review on the role of BH_4 in the nervous system, see reference (14).

It has long been proposed that BH_4 may be involved in cell proliferation, cell differentiation, and cell-mediated immunity. Such effects have been independently observed by several groups, however, the part of $BH_4$is complex and far from being clear.(2;64) Concerning the vascular system, BH_4 has been shown to stimulate in vitro angiogenesis in bovine aortic endothelial cells

by yet unclear mechanisms.(65) Nevertheless, it is of note that proliferative effects of several growth factors seem to depend on an obligatory elevation of intracellular BH_4 levels. This has first been shown for epidermal growth factor and nerve growth factor on the proliferation of rat phaeochromocytoma PC12 cells.(66) BH_4-involvement in cell cycle-dependent events by affecting c-Myc expression has been described very recently.(67) Furthermore, a critical role of BH_4 in the signaling cascade of vascular endothelial growth factor (VEGF) has been suggested (see below for details).

Of major importance for the effect of BH_4 in the vascular system, especially under pathophysiological conditions, is the strong antioxidative activity of reduced pterins;18 this unspecific function of BH_4 will be discussed in a later section of this review.

A modulatory role for BH_4 in the expression of of cyclooxygenase 2 has been shown for human mesangial cells. In these cells, supplementation with BH_4 potentiated cytokine-induced expression of cyclooxygenase 2 in an obviously NO-independent manner. If confirmed, these data corroborate the role of BH_4 as an important cellular mediator molecule in response to inflammatory stimuli.(68)

Finally, recent studies indicated an important role for BH_4 in regulation of human melanogenesis, for which tyrosinase (EC 1.14.18.1) that catalyzes the oxidation of L-tyrosine to the melanin precursor L-dopaquinone is the key enzyme. The rate of de novo melanin synthesis depends on supply of the substrate L-tyrosine or direct tyrosinase inhibition/activation. The BH_4 pathway is induced by UVB irradiation in relation to the degree of pigmentation, resulting in enhanced phenylalanine hydroxylase activity which may in turn supply L-tyrosine within melanocytes. In addition, BH_4 (but not oxidized forms) directly regulates tyrosinase activity by uncompetitive inhibition. The BH_4/tyrosinase inhibitor complex can be activated either by UVB photooxidation of BH_4, or by specific complexation with the 13 amino acid peptide a-melanocyte stimulating hormone (a-MSH). a-MSH, which is present in melanocytes, forms a specific and stable 1:1 complex with BH_4, thereby protecting the pteridine from photo-oxidation. Low levels of α-MSH activate phenylalanine hydroxylase, probably by removal of BH_4 from inactive tetramers of phenylalanine hydroxylase to yield active dimers. Since α-MSH levels are increased after UVB exposure, α-MSH has been postulated as a chaperone for BH_4 in the human epidermis. The redox state of BH_4 may influence not only pigmentation but also melanocyte survival and the process of keratinocyte differentiation. Pteridines are the principal molecules causing the fluorescence of depigmented skin used as a diagnostic tool in the depigmentation disorder vitiligo, and major disturbances in BH_4 metabolism and dependent enzymes have been found in vitiligo, eventually induced by epidermal H_2O_2 accumulation, but also in the Hermansky-Pudlak-Syndrome, a rare depigmentation disorder with autosomal recessive inheritance; however, the exact mechansims underlying these disturbances remain uncompletely understood at present.(69-73)

BH_4 AND THE VASCULAR SYSTEM

Recent years established the importance of BH_4 in the vascular system in health and disease. Most effects were linked to the L-arginine-NO-cGMP signaling system, and the vascular endothelium, a dynamic organ with vital secretory, metabolic, immunologic, and vasoregulative functions,(74;75) revealed to be central for BH_4 as a source and functional target of the pteridine. Soon after the discovery of an endothelium-derived relaxing factor (EDRF),(9) it was recognized that EDRF has identical properties as NO, a molecule known at that time to cause smooth muscle relaxation,(76) and EDRF was subsequently proposed to be either NO or a very labile nitroso compound readily releasing NO.(10;11;77) The ubiquity of the NO molecule has rapidly become clear, and it is now seen as a key mammalian metabolite for signaling in biological systems.(54;78-83)

NO maintains basal vessel tone by relaxing vascular smooth muscle cells, modulates vascular resistance in response to neurohormonal agents or physical forces, inhibits interactions between blood cells and vessel wall, is involved in the maintenance of vascular integrity, and influences proliferation and migration of smooth muscle cells.(74;84-86) In the physiologic state, vascular endothelial cells of different species including man (87-90) constitutively express eNOS protein. Endothelium-derived NO diffuses rapidly into subjacent vascular smooth muscle, where it binds to the heme iron complex of soluble guanylate cyclase and stimulates the production of cGMP and relaxation of vascular smooth muscle.(54;91) Attention has recently focused on the regulatory properties of BH_4 on eNOS activity.

BH_4 and the physiologic function of the vascular system

Endothelial cells. Human vascular endothelial cells express all enzymes for de novo BH_4 biosynthesis.(88;92-94) Cellular BH_4 turnover may be very rapid, as treatment with inhibitors of BH_4 biosynthesis deplete intracellular BH_4 levels within a few hours.(92;95) Cultured endothelial cells constitutively express GTPCH activity and synthesize small amounts

of BH_4, but prolonged culturing seems to cause a decline of intracellular BH_4 levels and a disappearance of GTPCH activity.(88) Human endothelial cells secrete the pteridine,(93;96) possibly vectorially into the basal direction.(97) Recently, evidence has been provided that human endothelial cells secrete not the fully reduced cofactor, but considerable amounts of 7,8-BH_2 and some biopterin.(96) This finding needs confirmation and is somewhat surprising since in all mammalian tissues that have been examined in vivo

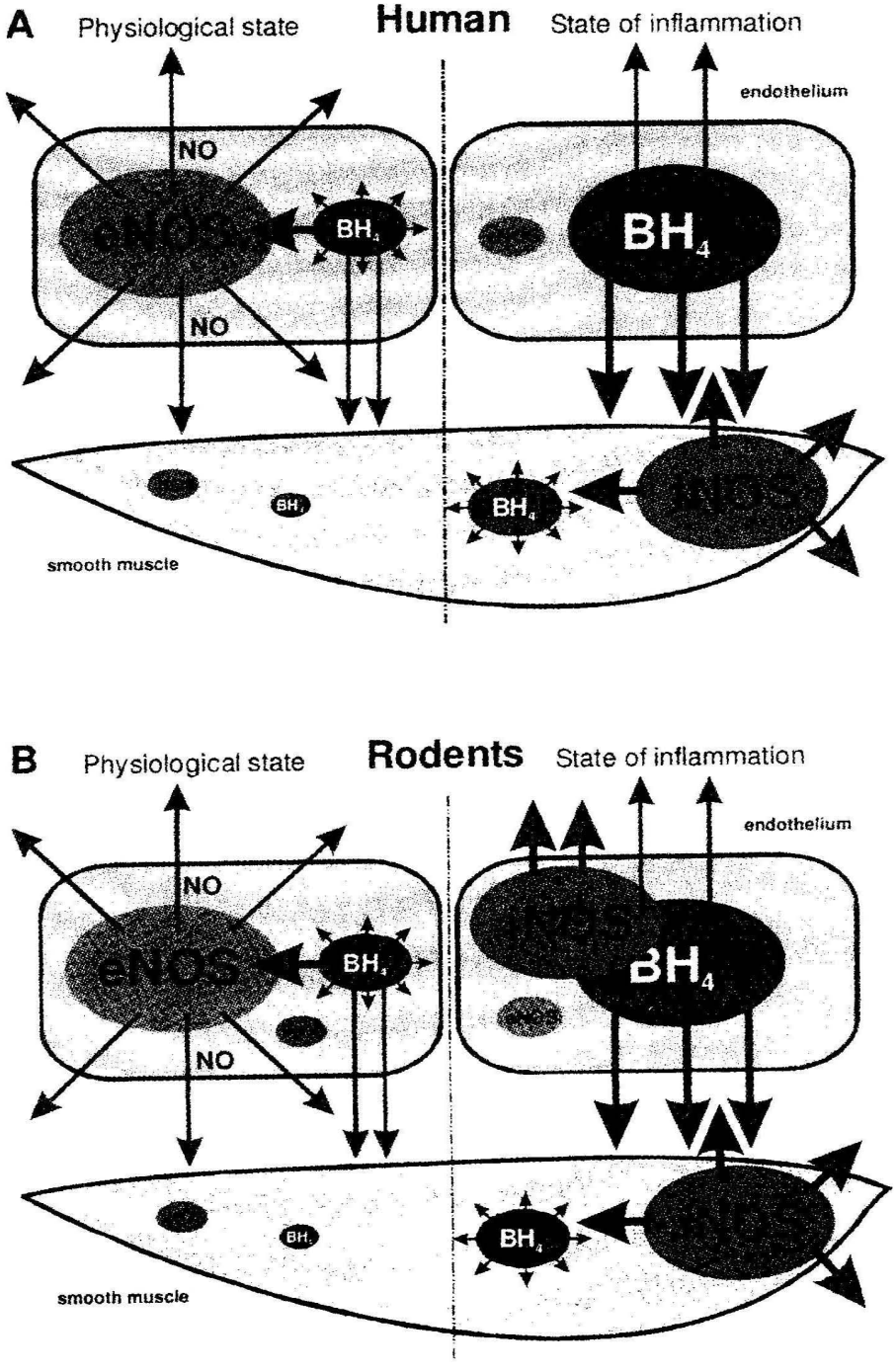

Figure 3. "Switch model". Regulation of NOS and BH4 synthetic activity under normal and inflammatory conditions in human (A) and rodents (B). See text for details.

85-100% of biopterin present is in the tetrahydro form.(98;99)

The signal transduction mechanism for and regulation of gene expression and the reason for the decrease of intracellular BH_4 during culture is unknown at present. However, GTPCH gene expression may be regulated by calcium influx,(100) and it is tempting to speculate that such a mechanism might be involved.

In cultured endothelial cells, exogenous sepiapterin increases intracellular BH_4. The GTPCH inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP) significantly reduces BH_4 levels within these cells which can be restored by sepiapterin.(92;95) These data show restoration of intracellular BH_4 via activation of the salvage pathway demonstrating that precursors of BH_4, such as sepiapterin, can be used as pharmacological tools to augment the pool of functional pteridine cofactor in vitro. However, the relative importance of this pathway in vivo remains unclear.

The bioavailability of BH_4 is pivotal for endothelial function, and intracellular BH_4 levels potentially regulate endothelial NO synthesis and vascular tone (88;95;101;102). eNOS activity in freshly isolated or cultured human endothelial cells is limited by intracellular BH_4.(88;96) Pretreatment of cultured cells with pro-inflammatory cytokines also largely increases eNOS activity and histamine-stimulated NO production, mainly due to increased GTPCH activity and subsequent elevated BH_4 levels.(88) In contrast, inhibition of BH_4 synthesis by DAHP reduces endothelial NO production.(92;95;96)

Smooth muscle cells. Functional de novo BH_4 biosynthetic and salvage pathways have also been found in human vascular smooth muscle cells.(103;104) However, GTPCH is barely expressed in cultured unstimulated human umbilical vein smooth muscle cells, only trace amounts of the pteridine are measured intracellularly, and no substantial amounts of BH4 are secreted.103 Since in cultured human smooth muscle cells neither iNOS nor eNOS mRNA is detected in the resting state,39;87 it can be assumed that BH_4 should have no direct NO-mediated relaxing effect on smooth muscle cells under physiologic conditions. This is corroborated by results from numerous studies which failed to demonstrate an influence of BH_4 on endothelium-independent relaxation. In addition, the availability of BH_4 obviously does not affect the reactivity of smooth muscle cells to NO.(19) Taken together, these data indicate that under physiological conditions in man, both NO and BH_4 production solely take place in the vascular endothelium but not in the underlying smooth muscle (Fig. 3A, left side).

Isolated vessels in vitro and in vivo experiments. BH_4 is present in intact arteries and veins, and about 60% of total BH_4 is located within endothelial cells.(102) This may however, not necessarily reflect true rates of production since endothelial cells secrete BH_4 that may be taken up by other cells. Indeed, rat smooth muscle cells readily take up BH_4 and sepiapterin,(39;105;106) and sepiapterin is also taken up by human vascular smooth muscle cells.(104) In line with these data are gene transfer experiments demonstrating that GTPCH activity and resulting BH_4 does not have to coexist in the same cell for the synthesized BH_4 to support iNOS activity.(107) Although formal proof for BH_4 uptake into human smooth muscle cells is lacking at the moment, it is conceivable to believe that BH_4 produced endogenously in vascular endothelial cells or elsewhere in the human body, traffics into smooth muscle cells of the vasculature.

Extending experiments on cultured cells, enhancement of endothelial NO synthesis by exogenous BH_4 could also be demonstrated in isolated vessels in vitro,(101) and relaxation was accompanied by dose-dependent increases of intracellular cGMP levels .(108)The effect of BH_4 on vessels in vitro seem to critically depend on experimental conditions, and additional BH_4 may even cause vessel contraction. (102;109) a result that has been explained by autoxidation of BH_4 and generation of superoxide anions.19 This hypothesis was corroborated by the observation that superoxide dismutase (SOD) enhanced endothelium-dependent relaxation in arteries with increased intracellular BH_4 levels.(102)

In vivo data suggest that under physiologic conditions levels of endogenous BH_4 are nearly saturating and barely a limiting factor for optimal or near optimal endothelial eNOS activity,(110-114) and only high doses of BH_4 may induce a marked local vasodilation.(97;115) In contrast to the requirement of rather high local BH_4 levels for vasodilating effects on the systemic circulation in healthy human subjects, myocardial blood flow may be increased at doses of BH_4 at which systemic hemodynamics are not affected suggesting that different human circuits may be differently susceptible to exogenous BH_4.(116)

Endothelium-dependent relaxation mediated by BH_4 under inflammatory conditions: proposal of the "switch" model

The expression of eNOS has traditionally been considered constitutive, but a multitude of regulating factors have been recognized.(86;117;118) Pro- and anti-inflammatory cytokines are probably of pivotal importance for the regulation of vascular tone under inflammatory conditions. Pro-inflammatory cytokines and endotoxin may transiently increase NO synthesis in human vascular endothelial cells (88;92;119) by indi-

rect regulation that includes enhanced synthesis of BH_4.(92) Nevertheless, there is increasing evidence that eNOS is downregulated under inflammatory conditions. Inflammatory stimuli decrease NO production and reduce eNOS protein and mRNA levels in cultured endothelial cells, (87-89;120-123) at least partially due to enhanced degradation rates of eNOS transcripts.(124-126) In contrast, anti-inflammatory cytokines may induce eNOS mRNA, protein, and activity.(127)

Inflammatory stimuli simultaneously induce the expression of iNOS mRNA and subsequent "high-output" NO production. Vascular smooth muscle cells from different species, including man, express iNOS mRNA and functional protein upon stimulation. Activation of the L-arginine-NO pathway and iNOS expression has been confirmed in human septic shock (128), although it has sometimes proved difficult to demonstrate a clear role for iNOS in human vessels in vivo for cytokine-induced vasodilation and a number of human experiments failed to show the consistent presence of iNOS under conditions in which this NOS isoform is readily induced in animals.(129) Important to note that whereas expression of iNOS has also been found in murine, swine, bovine, and rat endothelial cells,(123;130-134) human endothelial cells obviously express only eNOS but do not contain any iNOS.(88-90) Furthermore, no eNOS mRNA is detected neither under unstimulated nor under inflammatory conditions within human vascular smooth muscle cells.(39;87)

Culture of vascular cells with inflammatory stimuli increases expression of GTPCH, its enzymatic activity, and subsequently augments intracellular BH_4 levels, (88;92;105;135) whereas deactivating cytokines efficiently suppress BH_4 generation.(93) The increase in cellular BH_4 is mainly due to induction of de novo biosynthesis rather than via the salvage pathway using pre-existing dihydropterins.(94;136) In human vascular endothelial cells, most of the newly synthesized pteridine is secreted into the culture supernatant.(93) BH_4 biosynthesis is essentially required for induction of NOS activity, (38;105;137;138) and inhibition of BH_4 biosynthesis reduces iNOS activity in cytokine-stimulated cells.(38;105;138-141) Although expression of GTPCH and iNOS appears to be regulated coordinately (135;142-144) and BH_4 contributes to enhanced iNOS expression through stabilization of iNOS mRNA,(38;39) intrinsic BH_4 availability is a limiting factor for iNOS activity in many cell types including rat smooth muscle cells, (38;105;106; 137;139;145;146) rendering NO production of these cells susceptible to modulation of intracellular BH_4 levels. Treatment with inflammatory stimuli also enhances transcription of GTPCH mRNA and increases intracellular BH_4 levels in human umbilical smooth muscle cells, and small amounts of BH_4 are also secreted in the culture supernatant;(103) however it remains to be demonstrated whether BH_4 is also limiting for NO synthesis in human smooth muscle cells.

Activation of BH_4 biosynthesis has been confirmed in animal experimental sepsis, (144;147-150) and in some models biopterin apparently represents a more appropriate biochemical marker of septic shock than the determination of plasma NOx levels.(151) The regulation of BH_4 biosynthesis upon inflammatory stimuli in human vessels in vivo remains elusive, however, the induction of GTPCH in human veins challenged with cytokines has recently been confirmed.(115)

Taken the current knowledge together, a "switch" model is proposed to summerize changes of regulation of NO and BH_4 synthesis between physiologic and inflammatory conditions in the human vasculature (Fig. 3A). Although stimulatory cytokines may increase NO synthesis in human vascular endothelial cells, the concomitant dramatic decrease of eNOS mRNA makes this increase only transient, and a significantly reduced NO production is expected to occur after a certain time of ongoing inflammation. Thus, it must be concluded that NO could only account as an EDRF during short term but not during long term inflammation in man. Whereas eNOS mRNA and protein is downregulated, iNOS is induced, e.g. NO production switches and is no longer associated with eNOS but iNOS activity. Furthermore, the compartment responsible for NO production switches as well. Whereas eNOS is located in the vascular endothelium, iNOS is expressed in underlying smooth muscle cells. In contrast, the vascular endothelium continues to produce and secrete BH_4, and much higher amounts of BH_4 are secreted under inflammatory conditions. Although BH_4 synthesis is also induced in underlying smooth muscle cells upon inflammatory stimulation, these cells depend on exogenous BH_4 for full iNOS activity and readily take up the pteridine cofactor. BH_4 could therefore account as an EDRF during prolonged inflammation marking the third switch, i.e. the switch from NO as an EDRF under physiological to BH_4 as an EDRF under inflammatory conditions. As emphasized, species differences exist with respect to iNOS expression in vascular endothelial cells. Thus, the switch from exclusive endothelial to exclusive smooth muscle NO production is not generally valid, and in other species (e.g. in rat and mouse) iNOS may be expressed in both the endothelial and the smooth muscle compartment. As a result, both NO and BH_4 may coexist as EDRFs in these species under inflammatory conditions (Fig. 3B).

Experimental evidence for the switch model has been obtained both in vitro (87;123) as well as in vivo in rats.152 In healthy subjects in vivo, the early phase of inflammatory vasodilation may be mediated by eNOS activation due to increased amounts of BH_4, (115) but the question whether the switch from eNOS to iNOS takes place in later stages remains open.

Besides the induction of BH_4 biosynthesis in animal models of sepsis experimental data also indicate that inhibition of BH_4 metabolism or removal by hemoperfusion may prevent hemodynamic alterations suggesting the potential as novel therapeutic strategy against morbidity and mortality of septic shock.(153-156).

Oxidative stress: interplay of oxygen-derived free radicals with NO and BH_4

Endothelial functions are disrupted by a wide variety of perturbations. "Endothelial dysfunction" is believed to be an early event and important contributor in the etiology of several human vascular diseases, e.g. atherosclerosis, ischemia/reperfusion injury, and heart failure.(74;84) Besides loss of endothelial production and/or bioavailability of NO, a common denominator in these disorders comprises increased production of reactive oxygen intermediates (ROI), a class of molecules that includes superoxide anions, hydrogen peroxide, and hydroxyl radicals. Under certain conditions, production of ROI may outweigh endogenous antioxidant defense mechanisms, referred to as oxidative stress, and participate in the pathogenesis of cardiovascular disorders.(157) Many enzymatic systems are potential sources of ROI, which in turn can interact with vascular signaling systems and potentially oxidize any molecule in a cell, causing DNA nicking and disruption, lipid peroxidation, and protein cross-linking and degradation.(157-159)

The level of intracellular BH_4 is critical for the degree of ROI production by eNOS. Activation of purified eNOS in the presence of suboptimal levels of BH_4 results in uncoupling of oxygen reduction and arginine oxidation as well as generation of increased amounts of ROI,(51;52) a finding that was confirmed in vitro and in vivo.(160-163) The BH_4/7,8-BH2 ratio seems to control superoxide generation by eNOS as oxidized derivatives of BH_4 compete for binding and lead to enhanced ROI production implicating the redox state of the cofactor to be of central importance.(53) Therefore, decreased availability of BH_4 may cause a shift in the balance between the production of protective NO and toxic ROI and contribute to endothelial dysfunction and oxidative vascular injury. Interestingly, hydrogen peroxide itself may become a mediator of endothelium-dependent relaxations in states of BH_4 deficiency in vitro,(160) whereas superoxide causes endothelium-dependent contractions mediated in part by chemical inactivation of NO (see below).

Oxidative stress may contribute to impaired endothelium-dependent vasodilation through accelerated NO degradation. Superoxide reacts with NO in a nearly diffusion limited reaction yielding peroxynitrite at a reaction rate more than 3 times faster than that for superoxide with SOD.(164) Since the interaction of superoxide anions with NO is extremely rapid, this may even occur under physiologic conditions, and NO availability may become markedly reduced by superoxide anions and probably other radicals.

Peroxynitrite, a powerful oxidant with a relatively long half-life, is considered a primary reactive nitrogen species responsible for deletorious effects of NO. It is capable of hydroxylating and nitrating aromatic compounds, and inducing cellular injury by lipid peroxidation, DNA fragmentation, damage to proteins and plasma lipids, and depletion of important plasma antioxidants.(49)

As has been discussed, decreased availability of BH_4 may increase oxidative stress, and a decrease in BH_4 content in endothelial cells may indeed accelerate oxidative stress-induced endothelial cell death; (165)conversely, oxidative stress may contribute to decreased availability of functional pteridine cofactor by oxidative reactions. Under physiological conditions in vitro, peroxynitrite specifically oxidizes BH_4.(166) Peroxynitrite-catalyzed oxidation of BH_4 proceeds via two pathways from the quinoid 5,6-BH_2 intermediate. In one, a direct rearrangement to the more stable dihydro isomer 7,8-BH_2 occurs, and this isomer may be reduced back to BH_4. A large proportion of the quinoid 5,6-BH_2, however, loses its side-chain in the 6 position to form 7,8-dihydropterin, which can then be rapidly hydrated and converted to other species, including dihydroxanthoperin, thereby irreversibly losing cofactor function.(166) Finally, this may result in a vicious circle since limited bioavailability of BH4 uncouples L-arginine oxidation and increases eNOS-dependent superoxide production.

Autoxidation of BH_4. In vitro, BH_4 is susceptible to autoxidation in the presence of oxygen,(167-169) BH_4 may react with oxygen to yield oxidized pteridines and ROI.(102;170;171) Experiments with SOD suggest that superoxide is both a product of BH_4 autoxidation and accelerator of the autoxidative process.(170) The distribution of oxidized pteridines is nearly identical to those formed after oxidation with peroxynitrite, again indicating that ROI are most likely involved in this process leading to irreversible loss of cofactor function of the pteridine.(166) Evidence for autoxidation of

BH_4 has also been obtained in isolated vessels in vitro, and exogenous BH_4 regained the ability to stimulate endothelial NO synthesis anly after the addition of SOD.(102;109) Increased superoxide anions formed after BH_4 autoxidation could also interact with NO resulting in peroxynitrite generation and additional subsequent oxidative destruction of BH_4. These data imply that SOD may be critical for the effect of exogenous BH_4 on the vasculature, at least under experimental conditions. It is, therefore, noticable that in vascular endothelial cells increased endogenous BH_4 synthesis may be associated with simultanenous induction of SOD.(172) However, it is important to remember that all these findings have been obtained in vitro, and the relevance for in vivo conditions remains yet to be clarified.

Effects of exogenous BH_4. Beneficial effects of BH_4on oxidative stress include specific actions, i.e. reduction of ROI generation by eNOS as well as rather unspecific mechanisms. Several studies confirm the potential of BH_4, either in authentic form or given as sepiapterin, as antioxidant and scavenger of ROI, (173-178) see also reference (18) for review.

In line with the hypothesis of unspecific ROI scavenging is the observation that other pteridines, which share antioxidant and/or radical scavenging properties but no cofactor function with BH_4, also protect various cells against oxidative stress,(18;179-186) and the 7,8-dihydrostucture of the pteridines was recognized to indicate strong scavenging potency.(187-189)

Antioxidants: interplay of BH_4 with ascorbic acid (vitamin C) and folates?

Ascorbic acid. Lower dietary intake or plasma levels of ascorbic acid are associated with increased cardiovascular events. Conversely, ascorbic acid improves endothelium-dependent vasodilation in patients with coronary artery disease, risk factors for atherosclerosis, or chronic heart failure.(190) Prevention of oxidative modification of LDL, inhibition of leukocyte-endothelial cell interactions, and radical scavenging properties with prevented NO inactivation have been implicated,(191) however, ascorbic acid may not compete effectively with NO for superoxide at physiologically relevant concentrations,(192) and only results from studies using intraarterial ascorbic acid may be explained to a certain degree by superoxide scavenging. Another potential mechanism may depend on BH_4. In vitro, intracellular BH_4 levels are increased after treatment with ascorbic acid, (96;193;194) and low concentrations of ascorbic acid enhance NO synthesis only when BH_4 was omitted from cell lysate assays.(195) Likewise, pretreatment with ascorbic acid, which by itself dose-dependently augmented acetylcholine-mediated blood flow, abolished beneficial effects of BH_4 on endothelium-dependent vasodilation in vivo.(112) The action of ascorbic acid does not seem to be mediated by increased BH_4 biosynthesis, nor by modification of the pteridine affinity of eNOS, but may be due to chemical stabilization of BH_4 (96) thereby optimizing endothelial NO synthesis.

Folates. Low serum folate levels are associated with an increased risk of cardiovascular events,(196;197) traditionally ascribed to the cofactor function for enzymes of homocysteine metabolism. Mechanisms contributing to vasculopathy include promotion of endothelial dysfunction, impaired regulation of EDRF and related nitrogen oxides, and decreased availability of NO.(198-200) BH_4 may inhibit superoxide production induced by culturing of vascular cells with homocysteine.(201) Folate efficiently reduces plasma homocysteine levels and restores endothelial dysfunction induced by acute hyperhomocysteinemia.(202-205) In addition folate may improve endothelial function independently of its homocysteine-lowering effect that may include a reduction of ROI production by eNOS and other enzymes and increased availability of NO.(206;207) Interestingly, 5-methyltetrahydrofolate (5-MTHF) enhanced NO production and decreased superoxide production of partially pteridine-repleted but not of pteridine-free eNOS suggesting 5-MHTF to support BH_4 as cofactor of eNOS.(208) The exact intracellular mechanism remains unknown at present.

Endothelial dysfunction and atherosclerosis

Endothelial dysfunction occurs early during the pathogenesis of atherosclerosis, adds to alterations in vascular function and structure, and may be pivotal for the progress of atherosclerosis. Causes of endothelial dysfunction include elevated and modified low-density lipoproteins (LDL), free radicals caused by cigarette smoking, hypertension, diabetes mellitus, genetic alterations, elevated plasma homocysteine concentrations, infectious microorganisms, and other yet unidentified factors. A number of these disorders are associated with reduced synthesis/availability or increased degradation of endothelium-derived NO, and abnormal vasoreactivity is seen before morphological changes of atherosclerosis are visible. A growing body of evidence suggests increased production of ROI to be of pathophysiological importance contributing to inactivation of NO and endothelial dysfunction. (84;157;203;209-211)

In patients with cardiovascular risk factors or clinically manifest atherosclerosis, exogenous L-arginine normalizes endothelium-dependent vasodilation and diminishes lesion formation.(74;84) The exact mecha-

nism remain incompletely understood, but has mostly been attributed to an influence on eNOS activity. This is, at first glance, surprising since in vivo L-arginine concentrations appear to be well above the Km of eNOS for L-arginine,(212) and recently, it was suggested that effects independent of the role of L-arginine as substrate for eNOS may contribute as well.(212-216) A rapidly increasing number of studies demonstrates the potential of BH_4 to reverse endothelial dysfunction associated with risk factors leading to atherosclerosis or even in patients with manifest atherosclerosis and suggests that BH_4 may be of pivotal significance in the pathogenesis of this NO/oxidant imbalance.

Diabetes mellitus. Endothelial dysfunction is a common feature in experimental and clinical diabetes mellitus, and increased levels of ROI, release of an endothelium-derived constricting factor, and decreased endothelial NO synthesis are likely contributors to its pathogenesis.(210;217-219) Only little information is available concerning NOS activity, mRNA, and protein, but there is some evidence of normal or increased mRNA and protein levels despite impaired endothelial function due to a decrement of eNOS activity and increased endothelial superoxide generation.(220;221) Although in vitro NO synthesis was impaired, normal NOS activity was measured in cell homogenates under optimal conditions excluding an intrinsic defect in eNOS in these animals.(222)

Rat models of diabetes revealed an influence of the diabetic state on BH_4 metabolism. In these animals de novo biosynthesis of BH_4 as well as GTPCH and DHPR activities is reduced, and besides decreased BH_4 levels, increased concentrations of more oxidized forms are measured; in contrast, the BH_4 salvage pathway seems unaltered. Long-term oral treatment with BH_4 increases GTPCH and eNOS activity without affecting eNOS protein or mRNA expression, improves endothelium-dependent relaxation, and normalizes superoxide production, membrane lipid peroxidation, and binding activity of redox-sensitive transcription factors; beneficial effects have also been reported with the lipid-soluble pteridine derivative 6-methyl-BH_4. In addition, insulin stimulates the synthesis of BH_4 through activation of GTPCH in these animals, (221;223-226) and the endothelium-mediated vasorelaxation caused by insulin itself may be dependent on BH_4. (227) The suggestion that BH_4 biosynthesis might be reduced in diabetic conditions is supported by another study in LPS treated J774 macrophages.(228) Hitherto only limited information about human diabetes is available, but recent investigations indicate that BH_4 improves vascular endothelial dysfunction also in patients with type 2 diabetes mellitus.(113)

Smoking. Smoking has adverse effects on the vascular endothelium, and endothelial dysfunction in chronic smokers that includes an impairment of basal and stimulated NO production is well established. This impairment may be reversible on smoking cessation. Endothelial dysfunction of smokers is multifactorial and not entirely understood, but increased stress due to ROI is likely to be of central importance.(229) Immunostaining experiments suggest impairment of eNOS activity but not protein expression by smoking. An inhibition of eNOS by aromatic amines from the combustion of tobacco has been proposed.(230) Other contributing factors may include free radicals from smoke that may react to form peroxynitrite, and the induction of superoxide production as a result of autoxidation of polyhdroxyaromatic compounds;(112) these data suggest oxidation of BH_4 as the underlying cause of eNOS dysfunction, however, a formal proof for this hypothesis is lacking at the moment. BH_4 or sepiapterin improve endothelial dysfunction in human saphenous veins from smokers in vitro, and concomittantly increased production of nitrite/nitrate and cGMP indicate restoration of L-arginine-NO-pathway activity.(230) BH_4 also improves basal and endothelial-dependent vasodilation of chronic smokers in vivo through a rather specific effect, most probably the reduction of NOS-derived ROI, and not by a nonspecific antioxidant action.(112)

Hypertension. Endothelium-derived NO production has repeatedly been found to be impaired in essential hypertension. Oxidative stress plays an important role in the pathogenesis of hypertension, and besides other sources a dysfunctional eNOS has been identified as producer of superoxide.(203;210;231;232) In spontaneously hypertensive rats, exogenous BH_4 seemed not only to increase NO production but also to decrease levels of ROI although total biopterin levels in vessel walls were only slightly and non-significantly lower than in normotensive Wistar-Kyoto rats.(233) Only preliminary data are available on patients with essential hypertension. However, BH_4 increased endothelium-dependent vasodilation in forearms of such patients in a way that was abolished by an NOS inhibitor.(234)

Hypercholesterolemia. Endothelial dysfunction in hypercholesterolemia with impaired NO generation has been associated with increased endothelial ROI production which is at least part caused by eNOS itself.(210;235) In patients with familial hypercholes-

terolemia but without clinical signs of atherosclerosis, intraarterial administration of BH_4 restored endothelium-dependent vasodilation; the action was only transient, and discontinuation of BH_4 infusion resulted in reoccurrence of impaired NO activity within 20 minutes.(111) L-arginine is rate limiting in the L-arginine-NO pathway in hypercholesterolemia, and L-arginine alone improves NO-dependent vasodilation in such patients.(235) However, L-arginine causes no further improvement in the presence of BH_4 supplementation, indicating that functional BH_4 deficiency may contribute to the "L-arginine paradox" by decreasing the affinity of eNOS for L-arginine.(111)

Clinically manifest atherosclerosis. In severely atherosclerotic mice lacking the apolipoprotein E and LDL receptor genes, BH_4 slightly improved attenuated endothelium-dependent relaxation and, in combination with L-arginine, induced a pronounced enhancement of endothelium-dependent vasorelaxation whereas L-arginine alone was ineffective.(236) In nitrogylcerin-tolerant rats, BH_4 alone improved endothelium-dependent vasodilation suggesting that altered bioavailability of BH_4 may be of importance in this clinically relevant situation.(237) Interpretation of research on human atherosclerosis is hindered by the difficulty in obtaining true control vessels. However, there is evidence that basal NO release is impaired in atherosclerotic vessels,(238;239) and studies on human aortas indicate a downregulation of eNOS in endothelial cells overlying advanced atherosclerotic lesions.(240) These findings are supported by another study on arteries with advanced atherosclerosis obtained from patients undergoing carotid atherectomy or coronary bypass surgery in which eNOS protein and NO release was markedly diminished in luminal endothelial cells but not in endothelial cells of vasa vasorum inside the atherosclerotic plaque.(241) Consistent with these findings, differences in NO release observed in normal and atherosclerotic segments of explanted vein grafts are mirrored by focally reduced eNOS expression specific to atherosclerotic sites.(242) Despite the recurring observation of eNOS downregulation endothelial cells of atherosclerotic vessels, several studies demonstrated the potential of BH_4 to improve endothelium-dependent vasodilation in patients with manifest atherosclerosis; e.g. in isolated human coronary arterioles from patients with significant coronary atherosclerosis,(239) and in vivo in patients with coronary artery disease.(243) Preliminary data also indicate the potential of BH_4 to improve endothelium-dependent vasodilation and to reduce ROI generation in coronary arteries of patients with single or more cardiovascular risk factors but no significant coronary artery disease(244) and to enhance vasodilation in patients with atypical chest pain but angiographically normal coronary arteries.(245)

Recent immunohistochemical studies demonstrated the expression of iNOS in human and experimental atherosclerosis and a predominant presence in advanced atherosclerotic plaques,(240;246-249) however, no study hitherto performed has addressed the effects of BH_4 on iNOS in atherosclerotic plaques.

Saphenous vein grafts. In saphenous vein segments from patients undergoing coronary artery bypass graft operations, BH_4 roughly doubles actylcholine-induced vasorelaxation, indicating a dysfunctional endothelium already at the time of grafting.(250) Since in maturing vessel grafts endothelial NO synthesis may limit intimal hyperplasia, an improved supply of BH_4 could improve the patency of vessel grafts,(230) but long-term studies will be required test this hypothesis.

Taken together, BH_4 improves endothelial dysfunction associated with several risk factors for atherosclerosis or in manifest atherosclerosis. The biochemical background for a local functional BH4 deficiency in these disorders remains elusive, possible explanations include 1) increased oxidation of BH4 due to enhanced oxidative stress, and 2) decreased biosynthesis of the pteridine. The latter mechanism is supported by in vitro data demonstrating that oxidized LDL inhibits GTPCH gene expression in cytokine-activated cells.(251)

Endothelial dysfunction and chronic heart failure

Abnormalities in endothelial function with impaired endothelium-dependent vasorelaxation have also been described in patients with chronic heart failure, and ROI have been implicated in its pathogenesis.(210;252) To date, only preliminary and inconsistent data published in abstract form are available on the influence of BH_4 on the vasculature in chronic heart failure. Whereas in one study an effect of BH4 on endothelium-dependent vasodilation was lacking, in another an improvement was found, (253;254) and clearly further work is needed to clarify the role of BH_4 in this clinically important disease.

BH_4 and hypertension

The importance of eNOS for control of systemic and eventually pulmonary hypertension has been confirmed by eNOS knockout mice.(86) In the healthy vasculature, however, BH_4 requires rather high concentrations for hypotensive effects, as discussed earlier. Notwithstanding, BH_4 restores endothelial dysfunction associated with hypertension, and under certain experimental conditions the pteridine also has some

antihypertensive properties in animal models, e.g. in Male Sprague-Dawley rats fed high levels of fructose and uninephrectomized rats treated with deoxycorticosterone acetate and 1% NaCl in the drinking water (DOCA-salt rats).(225;255)

Involvement of BH_4 in ischemia-reperfusion (I/R) injury and "I/R tolerance"

Mechanisms of postischemic reperfusion (I/R) injury remain incompletely understood, but oxidative stress due to excess endothelial generation of ROI after the restoration of blood supply is thought to be of major importance.(158) In several animal models, exogenous sepiapterin, 6-methyl-BH_4, or BH_4 have shown beneficial effects on I/R injury.(256-261) In contrast, no study has investigated so far whether exogenous BH_4 also improves I/R injury in man.

Although evidence for a beneficial effect of BH_4 on I/R injury is sound, only an indefinite idea about underlying processes exists and several mechanisms likely contribute. BH_4 has potential activity as antioxidant and scavenger of ROI in endothelial cells, and this unspecific property may be involved. Furthermore, since experiments using inhibitors of BH_4 biosynthesis mimicked those from I/R experiments,(257;259) a decreased availability of functional pteridine cofactor is suspected in vessels exposed to I/R. As stated earlier, eNOS mediated ROI generation is triggered and controlled by decreased availability of BH_4. Conversely, exogenous BH_4 decreases superoxide levels by unspecific scavenging and reduction of eNOS dysfuntion.(51;52) Indeed, exogenous BH_4 may restore impaired eNOS function and increase cGMP production after I/R injury.(256;257;259)

The biochemical basis of eNOS impairment remains elusive. Giraldez et al. proposed a model to explain loss of eNOS activity occurring only after polonged I/R. They suggested intracellular acidosis to trigger titration of a critical amino acid (step 1) which would lead to further altered protein conformation with a partially unfolded protein (step 2). These two steps may be potentially reversible upon reperfusion. However, in a final step, the unfolded protein conformation would then be susceptible to proteolytic degradation, leading to the irreversible loss of enzyme activity.(262) Experiments with purified nNOS showed that stabilization of the dimeric state by BH_4 reduces inactivation by ROI.(36) Although this remains to be formally confirmed for the eNOS isoform, recently increased total eNOS protein but decreased immunoreactivity for dimeric eNOS after experimental I/R was demonstrated, and the latter was restored by additional BH_4.257 Being not only confirmative for intracellular depletion of BH_4 after I/R, these data also suggest depletion of BH_4 to be of central importance for the stepwise loss of eNOS activity.

An unresolved issue remains how I/R injury affects BH_4 metabolism and results in diminished functionality of the pteridine. Although decreased de novo BH_4 biosynthesis has been suggested,(257) the investigation was indirect; in fact, neither total biopterin contents nor expression of genes/enzyme activities involved in BH_4 biosynthesis/regeneration have ever been measured during or after experimental I/R injury. However, it is conceivable to assume that enhanced oxidative stress within endothelial cells may result in oxidation of BH_4 and loss of cofactor function, as outlined above.

Besides potentially disrupting and deleterious effects, I/R can also induce a series of events within hours or days that renders tissues more resistant to subsequent I/R insults. Recently, a critical dependence on activation and translocation of NF-kB for the development of "I/R tolerance" in endothelial cells was demonstrated.(90) Although both anoxia/reperfusion challenges were followed by transient increases in total eNOS protein, only the first was associated with increased oxidative stress, whereas after the second reduced oxidative stress was paralleled by increased NO production whith complete dependence on de novo BH_4 biosynthesis. It was suggested that NF-kB may contribute to the development of I/R tolerance by transactivating the GTPCH gene, and increased BH_4 levels would help to decrease subsequent I/R-induced cell damage.(90)

Vascular NOS gene therapy: requirement for BH_4

Recently, NOSs have emerged as potential candidates for vascular gene therapy to restore deficient endothelial NO production in a variety of cardiovascular diseases. NOS vascular gene transfer restores or augments NOS activity in the vessel wall, modulates vascular smooth muscle cell biology, and leads to regression of early experimental atherosclerosis.(263) Only very limited information is available at present about the role of BH_4 in NOS gene transfer. Almost all studies showed increased NO production after gene transfer without the addition of BH_4. However, some data indicate that BH_4 might be a limiting factor for the efficiency of NOS gene therapy. This is conceivable because 1) even under physiologic conditions BH_4 has the potential to regulate vascular NO production, and 2) smooth muscle cells which are likely target cells do not constitutively synthesize BH_4. In vitro, for example, additional sepiapterin significantly enhanced NO production in human vascular cells transfected with nNOS and porcine coronary artery smooth muscle cells transfected with eNOS.(104;264) In another

study, human iNOS was transfered into vascular cells and isolated blood vessels. Whereas transfected smooth muscle cells strongly dependent on exogenous BH_4 for NO synthesis, pulmonary endothelial cells obviously did not, and only porcine arteries transfected with human iNOS following balloon injury exhibited an increase in activity of NO-cGMP pathway over control vessels in a BH_4 dependent fashion clearly indicating that the level of functional BH_4 already present is a denominator whether exogenous BH_4 has any additive effect.(265) Today, functional BH_4 transfection by use of human GTPCH expression plasmids is technically feasible and reconstitutes near-maximal iNOS activity. Importantly, GTPCH and iNOS enzymes do not have to coexist within the same cells supporting the concept that BH_4 produced in one cells is accessible to neighbouring NOS expressing cells.(107) Consequently, first in vitro and ex vivo experiments confirm the potential of co-transfected BH_4 to enhance NO production achieved by gene transfer.(266;267) Although further verification is awaited, it is clearly indicated that supplementation with BH_4 may be required for efficient vascular NOS gene therapy, especially when the iNOS gene is transfected. It remains to be clarified whether cotransfection of GTPCH gene or direct administration of BH_4 is preferable to deliver BH_4 in these therapeutic settings.

Is BH_4 critical for synthesis and biological effects of VEGF-A?

The vascular endothelial growth factor (VEGF) family consists of several members of angiogenetic growth factors among those VEGF-A has been best characterized.(268) The effect of NO on VEGF-A remains a matter of some debate. It has convincingly been demonstrated, however, that NO upregulates VEGF-A synthesis.(269) Conversely, actions of VEGF-A are partly mediated by endothelium-derived NO,(270-278) and long-term exposure of endothelial cells to VEGF-A increases eNOS mRNA and protein levels.(279;280) First evidence for a modulatory role of BH_4 on cytokine-induced VEGF-A biosynthesis has recently been provided.(269) If confirmed, this finding may have some clinical significance as VEGF-A gene transfer is intensively studied as novel therapeutic approach to achieve angiogenesis in patients with symptomatic cardiovascular ischemic diseases.(281) In vivo data suggest a defective endothelial NO synthesis to be a limiting factor for the effect of VEGF-A.(282) Since in atherosclerotic vessels a functional BH_4 deficiency contributes to dysfunctional endothelial NO production, one may speculate that results of VEGF plasmid transfections could be critically dependent on local BH_4 levels, and that restoration of BH_4 (pharmacologically or by co-transfection with BH_4-encoding genes) could be a prerequisite for optimal treatment.

BH_4 and the pulmonary circulation

Pulmonary endothelial cell changes occur early in most clinical and experimental forms of acute lung injury.(283) Abnormalities of endothelial function associated with an imbalance in the expression of endothelium-derived vasoactive substances, including reduced expression of eNOS and subsequent diminished NO production, have been demonstrated in a number of pulmonary diseases such as primary and secondary pulmonary hypertension, chronic obstructive lung disease, cardiopulmonary bypass, and congestive heart failure. (284-287)

Surprising to note the minimal knowledge about effects of BH_4 in the lung. Although it is comprehensible to assume a regulation of pteridine synthesis similar to that in extrapulmonary tissues, experimental data to support this hypothesis are sparse. (59;149; 155; 288) So far, pathophysiologic conditions associated with local BH_4 deficiencies have not been described in the pulmonary circulation at all. Conversely, exogenous BH_4 improved pulmonary endothelial dysfunction following I/R injury by a mechanism involving the NO-cGMP pathway.(256) Further studies should clarify the role of BH_4 in other lung disorders and the potential to improve them. In a preliminary study it was demonstrated that BH_4 application by inhalation is feasible, well tolerated, and results in local resorption of BH_4 in the lung with subsequent increases in plasma and urinary BH_4 levels but without alteration of systemic hemodynamics.(114) Thus, inhalational BH_4 may offer a basic tool for investigations on restoration of pulmonary endothelial dysfunction in future, and it remains to be seen whether local administration of BH_4 by inhalation might be advantageous over systemic administration to achieve a therapeutic effect on endothelial dysfunction in the lung.

Conclusions

After more then a century of research on pteridine compounds it is now well established that BH_4 is of central importance for vascular functions in health and disease. BH_4 regulates vasomotion, modulates oxidative stress both by decreasing NOS-induced ROI generation and by unspecific ROI scavenging properties, is involved in effects of other pivotal antioxidants, and may be required in the signaling pathway of cellular mitogens and growth factors. A hitherto only uncompletely understood "BH_4-deficiency" has been implicated in a number of acquired vascular disorders, and

first clinical studies show promise with short-term BH_4-modifying treatment strategies. However, effects of long-term treatment with BH_4 are largely unknown and are eagerly awaited to answer the question whether BH_4, either supplemented as drug or via gene therapy, has the potential to prevent cardiovascular disease or to modify its clinical course.

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