

MINIREVIEW

Tetrahydrobiopterin and Nitric Oxide: Mechanistic and Pharmacological Aspects

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In previous minireviews in this journal, we discussed work on induction of tetrahydrobiopterin biosynthesis by cytokines and its significance for nitric oxide (NO) production of intact cells as well as functions of H₄-biopterin identified at this time for NO synthases (Proc Soc Exp Biol Med 203: 1–12, 1993; Proc Soc Exp Biol Med 219: 171–182, 1998). Meanwhile, the recognition of the importance of tetrahydrobiopterin for NO formation has led to new insights into complex biological processes and revealed possible novel pharmacological strategies to intervene in certain pathological conditions. Recent work could also establish that tetrahydrobiopterin, in addition to its allosteric effects, is redox-active in the NO synthase reaction. In this review, we summarize the current view of how tetrahydrobiopterin functions in the generation of NO and focus on pharmacological aspects of tetrahydrobiopterin availability with emphasis on endothelial function. Exp Biol Med 228:1291–1302, 2003

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Aromatic amino acid hydroxylases (i.e., phenylalanine, tyrosine, and tryptophan hydroxylase), all three of crucial importance for neurotransmitter synthesis, as well as glyceryl-ether monooxygenase were long believed to be the only (6R)-5,6,7,8-tetrahydro-L-biopterin (H₄-biopterin)-dependent enzymes. However, when nitric oxide synthase (NOS) was characterized some 15 years ago, H₄-biopterin was soon identified as one of its essential cofactors (1, 2). Discovering the multifaceted roles of NO for neurotransmission, endothelial function, and the immune response also fueled the interest in H₄-biopterin and regulation of its biosynthesis. Particularly intriguing was the finding that both H₄-biopterin and NO biosynthesis are under control of cytokines and that intracellular H₄-biopterin levels could modulate not only the activity of inducible NOS (iNOS, NOS2) but also of constitutive NOS, as was shown for the endothelial isoenzyme (eNOS, NOS3).

In previous reviews in this journal, we have focused on the regulatory network of cytokines, H₄-biopterin, and NO in various mammalian cell types as well as aspects of endothelial function and mechanism of the NOS reaction related to H₄-biopterin (3, 4). More recently, we reviewed the current knowledge on H₄-biopterin biosynthesis regulation as well as on pharmacological actions of H₄-biopterin regarding endothelial function, neuronal cell survival, and its role for development and differentiation (5). In addition, we summarized pharmacological aspects of H₄-biopterin analogs (6). Therefore, the present review will focus on new insights into the role H₄-biopterin is playing in the NOS reaction and impact of H₄-biopterin availability for certain NO-related pathological conditions, in particular endothelial dysfunction.

In addition to treatment of endothelial dysfunction, H₄-biopterin treatment of patients with H₄-biopterin responsive

mutations in the phenylalanine hydroxylase gene has recently received attention (7). Since a surprisingly high proportion (70%) of patients responded to H₄-biopterin treatment (8), and since H₄-biopterin can induce phenylalanine hydroxylase gene expression in the partially H₄-biopterin-deficient hph-1 mouse, it is assumed that in addition to its co-factor action, H₄-biopterin may increase expression of the phenylalanine hydroxylase gene (9). H₄-biopterin treatment allows these patients, who thus far had to consume a special diet low in phenylalanine, to live on a normal diet.

Mechanism of Stimulation of NO Synthases by H₄-Biopterin

Nitric Oxide Synthase Structure and Mechanism. The main synthetic pathway of NO in mammals is the enzymatic transformation of L-arginine into L-citrulline, catalyzed by NOS (EC 1.14.13.39). For overviews on various aspects of NOS enzymology and earlier studies not cited here, the reader is referred to recent reviews (10–13). The active site of NOS is formed by a heme-containing substrate-binding cavity, where L-arginine (Arg) and O₂ are converted to L-citrulline and NO. The electrons required for reductive O₂ activation are transferred from NADPH via the NOS-bound flavins FMN and FAD. All NOS isoforms are only active as homodimers. This dependence of NOS activity on the dimeric structure of the protein may be related to the essential role of “domain swapping”, (i.e., reduction of the heme in one subunit by electrons derived from the flavins in the other subunit) (14). The three mammalian isoforms, neuronal, endothelial, and inducible NOS (nNOS, eNOS, and iNOS, also designated as NOS1, NOS3 and NOS2) have distinct physiological functions, are localized to distinct cellular compartments, and are regulated by distinct signaling pathways. However, despite subtle differences in their biochemical properties, the basic mechanism of NO synthesis appears to be the same in all NOS isoforms, including the proteins cloned from lower organisms, such as invertebrates (15) or a myxomycete (16).

Generation of NO occurs in two discrete steps, with intermediate formation of *N*-hydroxy-L-arginine (NHA). NHA formation consumes one molecule of O₂ and two electrons. Conversion of NHA to L-citrulline and NO requires another molecule of O₂ and one more electron. The transformation of Arg to NHA is a cytochrome P450-type monooxygenase reaction. Accordingly, reduction of ferric heme [Fe(III)] followed by binding of O₂ yields the oxyferrous (superoxy ferric) complex [Fe(II) • O₂ ↔ Fe(III) • O₂⁻]. Further reduction by a second electron is thought to generate the superoxy ferrous/peroxy ferric state [Fe(II) • O₂⁻ ↔ Fe(III) • O₂²⁻]. In the presence of Arg, uptake of two protons will eventually result in formation of NHA and regeneration of ferric heme via several as yet poorly characterized intermediates.

The conversion of NHA to L-citrulline and NO is also a cytochrome P450-like reaction, but as the -NOH group undergoes atypical three- rather than two-electron oxida-

tion, the second step of NOS catalysis is thought to deviate from the P450 pathway after formation of the oxyferrous complex. Based on the fact that the overall stoichiometry of the second cycle requires only one electron, it is generally assumed that the heme is activated by bound NHA (i.e., that no further reduction by “exogenous” electrons is involved at this stage of the reaction).

Uncoupled Catalysis. One of the most striking differences between NOS and cytochrome P450 is that NOS requires H₄-biopterin as a co-factor. H₄-biopterin binds in the immediate vicinity of the heme at the dimer interface, interacting with residues from both subunits (17, 18). The conversion of Arg to NHA and of NHA to L-citrulline and NO both depend on the presence of H₄-biopterin. In the absence of substrate or pterin, NADPH oxidation by NOS is accompanied by formation of O₂⁻ and H₂O₂. It is not clear yet whether H₂O₂ is a genuine product of uncoupled NOS catalysis or if H₂O₂ derives exclusively from the disproportionation of O₂⁻ (19, 20). The extent of uncoupled catalysis varies between different isoforms. For nNOS, the rate of NADPH oxidation is unaffected by the presence of either of the two components that inhibit uncoupling when present together (i.e., substrate or pterin) (21). This is indicative of complete uncoupling in the absence of either of these two components. For iNOS, however, NADPH oxidation is strongly inhibited unless both Arg and H₄-biopterin are present (22).

Most studies point to the heme as the main site of uncoupled catalysis (23–28), despite some dissenting reports (19, 25). Most evidence suggests that uncoupling is only inhibited when H₄-biopterin and substrate are both present, although there are some reports demonstrating the ability of Arg or H₄-biopterin alone to suppress uncoupling (25, 26, 29). In the past few years it has become clear that the dependence of the coupled reaction on H₄-biopterin is not absolute. Some residual Arg oxidation (≤10%) occurs in the absence of pterin (30, 31). The transformation of NHA into L-citrulline in the absence of H₄-biopterin has also been reported (30, 32), although that reaction is most likely mediated by O₂⁻ (33). Both reactions purportedly yield NO⁻ instead of NO as a reaction product (30, 32).

Allosteric and Structural Effects of H₄-Biopterin and their Relevance to Catalysis. Since the discovery of H₄-biopterin as a co-factor of NOS, the function of the pterin has been a controversial issue, which was not settled before 1999 (i.e., after the previous update of this Minireview) (4). In aromatic amino acid hydroxylation, H₄-biopterin is involved in binding and activation of O₂. After hydroxylation of the substrate, the hydrated and 2-electron oxidized pterin dissociates and is enzymatically recycled to H₄-biopterin (34, 35). A similar role of H₄-biopterin in NO synthesis was ruled out soon, shifting the attention to structural and allosteric effects of pterin binding.

H₄-biopterin induces a shift of the NOS heme from low- to high-spin, and this effect correlates with enzyme activation (36, 37). H₄-biopterin substantially increases the

substrate affinity of NOS as was demonstrated in equilibrium binding studies (38), and, more recently, in activity assays as well (39, 40).

The best-characterized structural effect of H₄-biopterin is the stabilization of NOS dimers; for many years triggering NOS dimerization has been assumed to reflect the main function of the pterin. This effect is most striking for the inducible isoform, which forms the least stable dimers (41). Under certain conditions iNOS dimerization is strictly dependent on H₄-biopterin. However, dimeric forms of all three isoforms, including iNOS, can be obtained in the absence of H₄-biopterin (42, 43).

There are two other potentially beneficial effects of H₄-biopterin on NOS catalysis. First, H₄-biopterin binding prevents formation of inactive P-420 heme (44), and, secondly, the pterin may scavenge NOS-derived reactive nitrogen and oxygen species (40, 45). Both phenomena could contribute to prevent enzyme inactivation during catalysis. An interesting difference between iNOS and nNOS is that binding of Arg and H₄-biopterin increases the redox potential of the heme in iNOS but not in nNOS (46), an observation that may explain why iNOS shows less uncoupling than the neuronal isoform. Recent work using ultrafast time resolved absorption spectroscopy of eNOS suggested pterin-dependent formation of a new, non-heme NO binding site, which might control NO escape from the enzyme (47).

In summary, H₄-biopterin exhibits a range of allosteric and structural effects, presumably resulting from binding of the pterin in the dimer interface in close proximity to the heme and the substrate-binding site (17, 18). However, modulation of NOS activity through these effects appears to be relatively moderate, pointing to a distinct key function of H₄-biopterin in NO synthesis that is unrelated to allosteric effects.

A Novel Role for H₄-Biopterin as a 1-Electron Donor to the Heme. Although a classical function of H₄-biopterin in NO synthesis as a 2-electron donor has been definitely ruled out, studies with H₄-biopterin analogs suggested some kind of redox function of the pterin co-factor (for detailed discussion see References 4, 13). Although the natural 6R-(L-erythro-1', 2'-dihydroxypropyl) side chain of H₄-biopterin is required for high affinity to NOS (48), several tetrahydropterins, but no dihydropterins were able to support the reaction when used in high concentrations. 7,8-Dihydrobiopterin, on the other hand, bound fairly well to NOS (38) and was able to provoke allosteric effects identical to those seen with H₄-biopterin, but did not support the reaction (4, 13, 19, 22, 37, 45, 49–53). Since the electrochemical properties of 7,8-dihydropteridines preclude their participation in enzymatic redox cycling (54), these observations strongly suggested a redox function of H₄-biopterin in NOS. More recently we have shown that 5-methyl- H₄-biopterin (55) and other N5-substituted pteridines (50), which can undergo reversible 1- but not 2-electron oxidation (54), support NO synthesis, in agreement with the function of H₄-biopterin as a 1-electron donor suggested by the

EPR and light absorbance spectroscopic studies described below.

A major breakthrough in this puzzle was achieved in low-temperature optical studies, designed to detect short-lived intermediates formed in the reaction of reduced nNOS with O₂ (56). An intermediate observed in the absence of Arg or H₄-biopterin was identified as the oxyferrous complex, which decayed to the ferric state within a few minutes. In the presence of both Arg and H₄-biopterin, accumulation of the oxyferrous complex was prevented and stoichiometric amounts of NHA were formed. These results suggested that Arg and H₄-biopterin were essential for continuation of the reaction cycle beyond the oxyferrous complex, whereas in the absence of either substrate or pterin, this complex decayed to the ferric state and O₂⁻, giving rise to uncoupled NADPH oxidation (see previous discussion in this review). In this experimental set-up, there was only one electron (on the ferrous heme) available, but two electrons are required for Arg hydroxylation yielding NHA. This apparent inconsistency in reaction stoichiometry led us to suggest that the “missing electron” is provided by H₄-biopterin. Albeit without precedence, such a function of H₄-biopterin as 1-electron donor would explain both the strict pterin dependence of the NOS reaction under single turnover conditions and earlier observations pointing to a redox role of H₄-biopterin distinct from its classical function as 2-electron donor in aromatic amino acid hydroxylation (56). Similar spectroscopic observations were reported by others, although some groups arrived at different conclusions (20, 52, 57–60). The stoichiometric formation of NHA from L-arginine in single-turnover experiments was initially disputed (60, 61) but confirmed in later studies (20, 52, 62, 63).

Of course, the proposed novel function of H₄-biopterin as a 1-electron donor required experimental support. Early support for our hypothesis was provided by crystallographic studies showing that the pterin binding site of eNOS is lined with negatively charged residues that might help to stabilize a trihydropterin (BH₃^{*} • H⁺) intermediate (18). Definitive evidence for the ability of H₄-biopterin to donate an electron to the oxyferrous heme was provided one year later by detection of the trihydropterin radical using electron paramagnetic resonance (EPR) spectroscopy (62). This crucial observation was confirmed for all three NOS isoforms (20, 64, 65). In addition, it was shown that the pterin radical is indeed formed in the N5-protonated cationic state (65), as predicted by the crystal structure of the eNOS oxygenase domain (18), and careful kinetic studies have linked formation of the radical to decay of the oxyferrous state and product formation (20, 66). Intriguingly, the 5-methyl derivative exhibited a 3-fold faster rate of radical formation as compared to unsubstituted H₄-biopterin (66).

The reaction of NOS with the intermediate product NHA exhibits similar H₄-biopterin dependency as the initial hydroxylation of Arg (67). Single-turnover studies showed accumulation of the oxyferrous complex in the absence of NHA and/or H₄-biopterin but completion of the reaction

cycle with stoichiometric formation of L-citrulline when both NHA and H₄-biopterin were present (52, 60–62, 68). Because of these similarities and the lack of an obvious reason to assume that H₄-biopterin should not be able to donate an electron in the second cycle, we proposed that H₄-biopterin has an obligatory role as a transient electron donor in both reaction cycles (64, 68). However, this issue has remained controversial, in particular because the 1-electron stoichiometry of NHA oxidation might implicate that a second, H₄-biopterin-derived electron is not required (10, 60–62). Moreover, we and others failed to detect a pterin radical in the course of NHA oxidation (62, 63, 65).

However, although there is no net requirement for two electrons in the second reaction cycle, the oxyferrous heme must be reductively activated to allow O–O bond scission, and there is evidence that bound NHA is unable to activate the heme, even though it appears to serve as the ultimate electron donor (61, 68, 69). Based on the failure to detect a pterin radical in the course of NHA oxidation, it appears conceivable that the radical is rapidly reduced back to H₄-biopterin concurrently with L-citrulline formation by the “extra” electron available in the second reaction cycle, whereas the H₄-biopterin-derived electron is consumed to produce NHA in the first cycle, rendering the pterin radical sufficiently long-lived to be detectable by EPR (20, 52). In summary, the recent developments in the field have corroborated and provided a mechanistic explanation for the notion, originally suggested a decade ago (70), which indicates the main function of H₄-biopterin is to tightly couple NADPH oxidation to NO synthesis.

The presence of H₄-biopterin is necessary but not sufficient to prevent uncoupling of the NOS reaction. To explain the additional requirement for an amino acid substrate (Arg or NHA), it has been proposed that reduction of oxyferrous heme to Fe(II) • O₂[−] by H₄-biopterin would result in uncoupled decay to ferric heme and H₂O₂ in the absence of substrate (20). Although this hypothesis cannot be ruled out, an alternative scenario is also feasible. On the basis of electrochemical studies, H₄-biopterin is expected to be a poor 1-electron donor (54). Thus, albeit kinetically fast because of the close proximity of the pterin to the heme, electron transfer from H₄-biopterin to the oxyferrous complex may be energetically unfavorable. Accordingly, the species Fe(II)O₂ ••• H₄-biopterin and Fe(II)O₂[−] ••• BH₃[•] may be in rapid equilibrium with the tetrahydropterin state predominating. In that case uncoupling will still occur, unless substrate is present to pull the reaction forward along the catalytic cycle (13). In addition, this hypothesis explains why H₄-biopterin cannot provide the first electron in the NOS reaction cycle, a redox reaction that would involve reduction of ferric heme against a pronounced potential gradient. Figure 1 summarizes our views on the catalytic mechanism of NO synthesis.

Modulation of Nitric Oxide Synthase Product Distribution by H₄-Biopterin. Since H₄-biopterin prevents uncoupling of NADPH oxidation from NO synthesis, decreasing concentrations of the pterin will result in in-

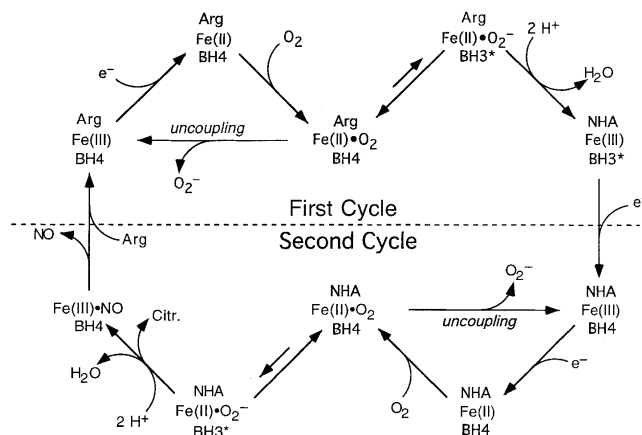


Figure 1. Proposed role of H₄-biopterin in NO synthesis. Reduction of ferric heme is followed by oxygen binding. The oxyferrous complex will yield ferric heme and superoxide in the uncoupled reaction, unless H₄-biopterin and substrate (Arg or NHA in the first and second cycle, respectively) are both present to allow continuation of the cycle. In the reaction with Arg, the BH₃[•] radical is detectable by EPR in single turnover studies, whereas H₄-biopterin may be rapidly regenerated in the reaction with NHA.

creased O₂[−]/H₂O₂ and concomitantly decreased NO formation. Indeed, there is ample evidence that H₄-biopterin deficiency provokes NOS-derived O₂[−]/H₂O₂ production in conditions of oxidative stress (see below). However, alternative ways of how H₄-biopterin might modulate the outcome of the NOS reaction are also feasible. NOS was found to be only half-saturated with H₄-biopterin (39, 61, 63). This phenomenon has been explained by anticooperative binding of H₄-biopterin to the NOS dimer (36). If this hypothesis holds, NOS may catalyze the formation of equal amounts of NO and O₂[−] over a wide range of pterin concentrations (13, 68, 71). Consequently, as first noted in 1995 (72) NOS may function as a NO/O₂[−] (peroxynitrite) synthase under certain conditions. In standard enzyme incubation mixtures, NO is detected as reaction product only in the presence of the autooxidation resistant N5-substituted pteridines: For this property, these derivatives may constitute a class of potentially useful enzyme activators. However, the low binding affinity of the lead compound, 5-methyl-H₄-biopterin, so far has precluded its use in pharmacological studies (50, 54, 55, 71). More recently, a new twist was added to the plot when it was demonstrated that the simultaneous generation of NO and O₂[−] by pterin-subsaturated NOS resulted in the efficient formation of S-nitrosoglutathione (GSNO) in the presence of physiological concentrations of GSH (73). While peroxynitrite formation is expected to exhibit deleterious biological effects, nitrosothiols may serve as relatively long-lived stores and/or transport forms of bioactive NO (10).

H₄-Biopterin and Endothelial Dysfunction

Effects of H₄-Biopterin Treatment. The potential involvement of H₄-biopterin in endothelial dysfunction has recently received much attention. Since lack of H₄-biopterin causes an alteration of the ratio of superoxide to nitric oxide

by the purified enzyme, and since endothelial dysfunction was found to be correlated with decreased NO and increased superoxide production, partial H₄-biopterin deficiency is an attractive hypothesis for the cause of impaired vasodilatory responsiveness in certain clinical settings such as the insulin-resistant state, diabetes, or atherosclerosis. In addition to the concentration of H₄-biopterin, the ratio of 7,8-H₂-biopterin to H₄-biopterin might be important, since this ratio has been shown to control superoxide release from endothelial NO synthase (53). Indeed, depletion of H₄-biopterin in canine (74) and rat (75) arteries as well as in rats *in vivo* (76) led to endothelial dysfunction within a few hours. Apart from these investigations, the hypothesis of H₄-biopterin deficiency as cause for impaired vasodilatation was tested primarily by the effects of H₄-biopterin or sepi-

apterin treatment on the responsiveness of blood vessels to stimulation by agents acting via NO (e.g., acetylcholine). NOS has a more than 10-fold lower K_M for H₄-biopterin compared with aromatic amino acid hydroxylases, and may therefore be the primary target for administered H₄-biopterin. It cannot be excluded, however, that biosynthesis of catecholamines, which have vasoconstrictive properties, might be potentially increased by H₄-biopterin. This issue, however, has not yet been addressed experimentally in attempts to manage vascular dysfunction. Influence of H₄-biopterin on catecholamine biosynthesis might be of minor importance in these settings, since H₄-biopterin consistently improves endothelial function in a number of vascular diseases. Table I summarizes these attempts of H₄-biopterin treatment regimens. With one exception (77), H₄-biopterin

Table I. Effect of Application of Agents Modulating H₄-Biopterin Concentrations in Various Experimental and Clinical Settings

Species/study design	Effect of H ₄ -biopterin (related) treatment	Reference
Dog, artery	H ₄ -biopterin depletion leads to endothelial dysfunction	74
Human, hypercholesterolemia	H ₄ -biopterin plus L-arginine restore impaired vasodilatation	130
Human, hypercholesterolemia	H ₄ -biopterin improves vasodilatation	131
Human, pig, atherosclerosis	Sepiapterin improves vasodilatation	132
Human, saphenous vein	H ₄ -biopterin improves vasodilatation	133
Human, coronary heart disease	H ₄ -biopterin attenuates endothelial dysfunction	134
Human, normo- or hypertensive	H ₄ -biopterin augments vasodilatation in both groups	77
Human, vasospastic angina	H ₄ -biopterin improves coronary function, but not spasm	135
Human, chronic heart failure	H ₄ -biopterin improves vasodilatation	136
Human, long-term smokers	H ₄ -biopterin improves vasodilatation	137
Human, chronic smokers	H ₄ -biopterin, but not H ₄ -neopterin, attenuates endothelial dysfunction	80
Human, EAhy926 cells	GTP-cyclohydrolase I gene transfer augments eNOS	82
Mouse, Apo-E deficient	Sepiapterin improves vasodilatation	138
Mouse, Apo-E deficient, + eNOS	H ₄ -biopterin counteracts damage of eNOS overexpression	81
Mouse, Apo-E deficient	Ascorbate lowers H ₂ -biopterin and improves vasodilatation	90
Mouse, partially H ₄ -biopterin deficient	H ₄ -biopterin counteracts catalase sensitivity of vasorelaxation	113
Rat, spontaneously hypertensive	H ₄ -biopterin lowers O ₂ ⁻ /H ₂ O ₂ and restores NOS activity	109
Rat, glucocorticoid-treated	Sepiapterin restores vasorelaxation	106
Rat, mouse, deoxycorticosterone-treated	H ₄ -biopterin blunts deoxycorticosterone-induced hypertension	107
Rat, cerebral arterioles	Nicotine impairs vasodilatation; H ₄ -biopterin counteracts	139
Human, diabetes mellitus	H ₄ -biopterin improves vasodilatation	140
Human, diabetes mellitus	H ₄ -biopterin corrects increased NADPH oxidation	104
Mouse, spontaneously diabetic	H ₄ -biopterin and sepiapterin improve vasodilatation	141
Mouse, mesangial cells	H ₄ -biopterin attenuates inhibition of iNOS induction by glucose	142
Rat, insulin-treated, fructose-fed	Insulin-resistant state leads to lower H ₄ -biopterin, higher H ₂ -biopterin	99
Rat, fructose-fed	Oral H ₄ -biopterin prevents endothelial dysfunction	100
Rat, fructose-fed	H ₄ -biopterin improves vasodilatation	143
Rat, diabetic, aortic rings	6-methyl-H ₄ -pterin augments relaxation	144
Rat, diabetic, arterioles	Sepiapterin improves vasodilatation	75
Rat, PC-12 cells	H ₄ -biopterin protects from NO-mediated glucose toxicity	145
Pig, coronary arteries	Sepiapterin restores vasodilatation after reperfusion	146
Mouse, rat, primary neurons	Sepiapterin protects against vulnerability due to GSH depletion	147
Rat, primary neurons	Sepiapterin protects against toxicity due to GSH depletion	148
Rat, neuronal cultures	H ₄ -biopterin depletion causes vulnerability to hypoxia	149
Rat, human cells; ischemia	H ₄ -biopterin protects against ischemia-reperfusion damage	150
Rat, isolated heart	H ₄ -biopterin protects against ischemia-reperfusion damage	91
Rat, renal allografts	H ₄ -biopterin increases NO; diminishes tyrosine nitration	151
Human, endothelial cells	Sepiapterin blunts ascorbate-mediated increase of NO production	85
Human, endothelial cells	Ascorbate stabilizes H ₄ -biopterin and increases eNOS activity	87
Human, endothelial cells	Ascorbate increases H ₄ -biopterin and increases eNOS activity	88
Human, placenta	Ascorbate stabilizes H ₄ -biopterin and thus activates eNOS	93
Pig, endothelial cells	Ascorbate increases H ₄ -biopterin and increases eNOS activity	86

shows effects only in the pathological state, but not in healthy controls. In the conditions shown in Table I, the mechanism of vasodilatation downstream of NO production, however, appears to be intact and not affected by H₄-biopterin treatment as judged by the responses of the vessels or cells to, for example, glyceryl trinitrate. H₄-biopterin appears to be an efficient drug in restoring endothelial dysfunction in a surprisingly wide range of experimental settings (Table I). See also previous reviews by Katusic (78), and Tiefenbacher (79). Abrogation of the effects of tetrahydrobiopterin by NOS inhibitors confirms that the effects observed are mediated by NOS. Further confirmation that H₄-biopterin exerts its action via NOS comes from the observed stereospecificity of the effect. In contrast with H₄-biopterin, H₄-neopterin had no effect on forearm bloodflow in chronic smokers (80). Since H₄-neopterin has chemical properties similar to H₄-biopterin, but does not bind to NOS due to its different side chain at C6 of the pterin ring (48), this finding strongly suggests that H₄-biopterin exerts its action on vasodilatation primarily by acting as co-factor of NOS rather than by chemical reactions. A striking example for the importance of H₄-biopterin for endothelial function is the attempt to correct vascular dysfunction in ApoE-deficient mice by overexpression of eNOS (81). Overexpression of eNOS in these animals had a detrimental effect that could be corrected by administration of H₄-biopterin. On the other hand, overexpression of GTP-cyclohydrolase I, the key enzyme of H₄-biopterin biosynthesis, in an endothelial type cell line by an adenoviral system dramatically increased intracellular H₄-biopterin levels and NO production by these cells (82).

Vascular Effects of Ascorbate—often Mediated by H₄-Biopterin Stabilization. In addition to H₄-biopterin, ascorbate has also been shown to restore impaired vasodilatation in a variety of clinical settings (83, 84). Experiments with endothelial cells in culture demonstrated that ascorbate leads to increased NO production, an effect that could be blinded by sepiapterin treatment, suggesting that the ascorbate effect was mediated by H₄-biopterin (85). Subsequently, increased H₄-biopterin was detected in cells treated with ascorbate (86–88). Detailed investigation of H₄-biopterin biosynthetic activities and content of the various oxidation states of biopterin in cells and culture media demonstrated that ascorbate increased intracellular H₄-biopterin and thereby NO synthesis via a chemical stabilization of H₄-biopterin (87). In agreement with the above-mentioned *in vitro* findings, pretreatment with ascorbate abolished the effect of H₄-biopterin on forearm bloodflow in chronic smokers (80). In addition, ascorbate, like H₄-biopterin in diabetic subjects, restores endothelium-dependent vasodilatation impaired by acute hyperglycemia in healthy humans (89). Ascorbate treatment of ApoE-deficient mice restored impaired vascular eNOS activity and lowered vascular 7,8-H₂-biopterin levels (90). On the other hand, in ischemia-reperfusion injury, H₄-biopterin, but not

ascorbic acid or superoxide dismutase, protects from organ damage (91).

Figure 2 summarizes the current knowledge of how enzymatic activities and ascorbate are thought to influence interconversion between different oxidation states of H₄-biopterin. In addition to the NOS reaction, which generates a H₃-biopterin radical cation (65) (for detailed discussion see previous section), a neutral H₃-biopterin radical is formed upon reaction of H₄-biopterin with various radicals (92), which can be recycled to H₄-biopterin by ascorbate. Alternatively, the H₃-biopterin radical can disproportionate to the quinonoid 6,7-[8H]-H₂-biopterin, which is also reduced by ascorbate to H₄-biopterin (93). In addition, the quinonoid 6,7-[8H]-H₂-biopterin is the product of H₄-biopterin catalyzed aromatic amino acid hydroxylation followed by cleavage of the 4a-hydroxy intermediate by carbinolamine dehydratase (34, 94). In contrast with the NOS reaction, theoretical considerations of aromatic amino acid hydroxylation do not require the formation of a pterin radical (35). The quinonoid 6,7-[8H]-H₂-biopterin can be enzymatically reduced to H₄-biopterin by dihydropteridine reductase (DHPR). Once the quinonoid 6,7-[8H]-H₂-biopterin

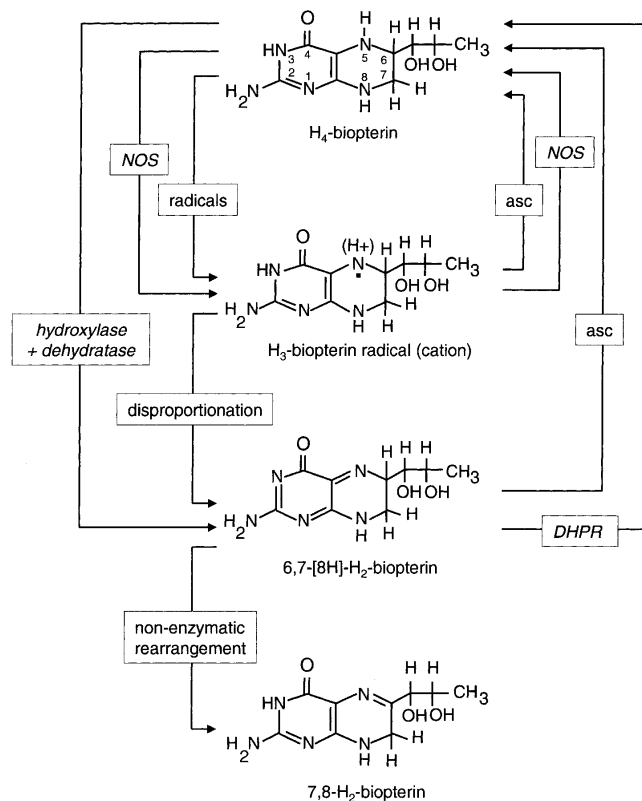


Figure 2. Pathways for the interconversion between different oxidation states of H₄-biopterin. While the H₃-biopterin radical formed in NOS has spectra best fitted by a cation (65), chemical oxidation leads to a neutral species at neutral pH (92). 7,8-H₂-biopterin can be converted to H₄-biopterin by dihydrofolate reductase. This pathway, however, is omitted from the figure since it appears not to be relevant physiologically due to the *K_M* in the 50 μM range, unless high concentrations of 7,8-H₂-biopterin are reached in cells (e.g., upon sepiapterin treatment). Asc, ascorbic acid; DHPR denotes dihydropteridine reductase. See text for details.

has undergone non-enzymatic rearrangement to 7,8-H₂-biopterin, it appears to be lost as co-factor and is excreted by cells to the culture medium (87). Although it is known that 7,8-H₂-biopterin can serve as substrate of dihydrofolate reductase yielding H₄-biopterin, this reaction appears to be of little importance unless high concentrations of 7,8-H₂-biopterin occur (e.g., by treatment with sepiapterin), which is efficiently converted to H₄-biopterin by cells due to the sequential action of sepiapterin reductase and dihydrofolate reductase. A reason for this behavior might be the high K_M value of dihydrofolate reductase (DHFR) for 7,8-H₂-biopterin (43–88 μ M) (95, 96). In contrast, K_M values for the physiologically relevant reductions of 7,8-dihydrofolate by dihydrofolate reductase and 6,7-[8H]-H₂-biopterin by dihydropteridine reductase are 0.1 μ M (95) and 0.1 μ M to 0.3 μ M (97), respectively.

Mechanisms Leading to Partial H₄-Biopterin Deficiency in Diabetes. The possible mechanisms leading to the suspected H₄-biopterin deficiency have received less attention than the above-mentioned effects of H₄-biopterin administration. In insulin-resistant patients, impaired vasodilatation was found paralleled by a decreased H₄-biopterin to H₂-biopterin ratio, as well as decreased dihydropteridine reductase activities (98). Similar observations have been made in diabetic rats (99, 100). The mechanisms of how hyperglycemia leads to H₄-biopterin deficiency and endothelial dysfunction are only poorly understood. It has been known for a long time that insulin can augment GTP-cyclohydrolase I activities (101), and a more recent study confirmed that insulin-induced vasodilatation depends on H₄-biopterin biosynthesis (102). These mechanisms may be impaired in the insulin-resistant state. Remarkably, high glucose leads to endothelial dysfunction already 6 hrs after increase in concentration, and this effect can be blocked by inhibition of protein kinase C (103, 104).

Other Conditions Leading to Impaired Vasodilation Paralleled by Partial H₄-Biopterin Deficiency. In addition to diabetes, partial H₄-biopterin deficiency associated with endothelial dysfunction has been observed in other conditions such as 17 β -estradiol depletion (105), glucocorticoid (106) and deoxycorticosterone (107) treatment, hypercholesterolemia (108), and in the hyperphenylalaninemic mouse (hph-1) mutant (109). Ovariectomized rats show impaired vasodilatation, which can be restored by H₄-biopterin (105). Similarly, glucocorticoid-induced hypertension was found to be associated with decreased NO-dependent vasodilatation in aortic rings, which could be corrected by sepiapterin. In contrast with diabetes, where dihydropteridine reductase appears to be affected, GTP-cyclohydrolase I expression was found to be lowered by dexamethasone in these animals (106). Deoxycorticosterone treatment, on the other hand, leads to oxidation of tetrahydrobiopterin, uncoupling of eNOS, and hypertension (107). When rabbits were fed with hyperlipidemic chow, this resulted in highly increased cholesterol and dramatically lowered H₄-biopterin levels. Remarkably, sepiapterin could not

reverse impaired vasodilatation in aortae cultured from these animals (108). The anticipated effect might have been achieved, however, by coincubation with superoxide dismutase, since in cultured dog arteries, sepiapterin could augment cGMP levels only when administered together with superoxide dismutase (110). See also comment in (111). Hph-1 mice are mutants with a partially reduced GTP-cyclohydrolase I activity, leading to lowered H₄-biopterin levels that can be corrected by administration of H₄-biopterin (112). These animals show altered responsiveness of vasorelaxation to catalase and superoxide dismutase when compared with normal control animals, a difference that vanishes after H₄-biopterin treatment (113).

In addition to partial H₄-biopterin deficiency, another attractive mechanism of how peroxynitrite, high glucose, or hypercholesterolemia leads to dysfunction of endothelial NOS is the removal of Zn(2⁺) from eNOS, which resulted in decreased NO and increased superoxide production (114). Although this has not yet been addressed experimentally, it seems possible that H₄-biopterin, which stabilizes particularly eNOS dimers (41), might counteract the mechanisms removing the zinc and thereby confer its protective action. Increased vascular oxidant stress seems partially to be due to an increased expression of NADPH oxidase, as has been shown in experimental left ventricular hypertrophy (115) and in diabetes (104). Moreover, electron spin resonance measurements revealed increased xanthine and NADPH oxidase activities in coronary arteries from patients with coronary artery disease (116). Increased production of superoxide by NADPH oxidase might lead to oxidation of tetrahydrobiopterin and NOS uncoupling, an effect that was overcome by tetrahydrobiopterin supplementation (107). Taken together, the diverse indications for successful H₄-biopterin administration in various conditions remain intriguing (Table I). Lack of effect of H₄-biopterin has been reported only in exceptional cases, such as impaired endothelial regulation of ventricular relaxation in cardiac hypertrophy in the guinea pig (115).

Catabolism of H₄-Biopterin. Catabolism of H₄-biopterin (117–119) as well as its renal excretion (120) has been studied in some detail in rats more than two decades ago by Heinz Rembold *et al.* Using ¹⁴C-labeled H₄-biopterin, these authors found that only a small portion (10%–15%) of the administered H₄-biopterin reappeared as fully oxidized biopterin. Most of the administered H₄-biopterin lost its side chain at C6 to yield 7,8-dihydroxanthopterin and xanthopterin (11%–19%), and was further deaminated at C2 to yield 6-hydroxylumazine (27%–28%). Other catabolic products of tetrahydrobiopterin identified include isoxanthopterin and leucopterin. While these degradation reactions can occur to some extent by chemical reactions in neutral solution, they have been shown to be greatly assisted by enzyme activities present in tissue homogenates. One of these activities contributing to degradation has been identified as xanthine oxidase. To our knowledge no comparable studies have been performed in humans thus far. While the

catabolic reactions might be largely similar in humans and rats, the comparatively lower concentrations of lumazines in human urine (121) may indicate that enzymatic activities yielding deamination of C2 of the pterin ring might be less active in humans than in rodents.

Pharmacological Effects of Inhibitory H₄-Biopterin Analogues

One of the best-studied NOS inhibitory pteridines is 4-amino-H₄-biopterin (6, 13), a compound in which the keto function at C4 is replaced by an amino group. This structural analogue of the well-known inhibitor of tetrahydrofolate reductase, methotrexate, proved to be a very potent H₄-biopterin-competitive inhibitor of NO synthesis, even though this compound is virtually identical to H₄-biopterin with respect to its electrochemical properties and allosteric effects (e.g., low-to-high-spin transition of the heme and stabilization of NOS dimers) (4, 13). In addition, the orientation of this analogue in crystals of iNOS oxygenase domain exactly resembles that of the natural co-factor H₄-biopterin (122). Thus, the mechanism of inhibition of NOS by 4-amino-H₄-biopterin differs from the mechanism of inhibition of dihydrofolate reductase by methotrexate, which binds in an altered orientation to the active center of dihydrofolate reductase (123). An attractive explanation of the mechanism of inhibition of NOS by 4-amino-H₄-biopterin is the altered electrostatic potential at N3 (124), which is the position of the molecule interacting with the heme (17). While 4-amino-H₄-biopterin inhibits all three purified isoforms of NOS in the micromolar range, in cultured cells as well as in aortic strips a selectivity for inhibition of the inducible isoform of NOS is observed (125, 126). Phenylalanine hydroxylase, in contrast, is not inhibited by 4-amino-H₄-biopterin in concentrations up to 1 mM. In a rat model of septic shock, a single dose of 10 mg/kg of 4-amino-H₄-biopterin was able to save the animals from the lethal effects of endotoxin (127). In a murine model of cardiac allograft rejection, the 4-amino H₄-biopterin was as efficient as high-dose cyclosporin A treatment in suppressing allograft rejection (128). In addition to 4-amino-H₄-biopterin, a series of other pterin-based compounds has also been investigated for inhibition of NOS (129).

Depending on their effects on the redox potential of the heme (22), some substrate-based NOS inhibitors, like the N^G-methyl derivative of Arg, attenuate NO synthesis but do not block NADPH-dependent O₂ activation. This type of compound is expected to switch the outcome of the NOS reaction from NO to O₂⁻, casting doubt on their clinical benefits. Therefore, it appears essential to clarify how pterin-site inhibitors affect NOS uncoupling before considering this class of compounds for clinical use. Current evidence suggests that some pterin derivatives do indeed support the uncoupled reaction (46, 53), but definitive conclusions await detailed comparative studies evaluating the effects of a wide variety of compounds on the catalytic functions of the three NOS isoforms.

Abbreviations: For a proposal of nomenclature of pteridine compounds compare Reference 152. H₄-biopterin, BH₄: 5,6,7,8-tetrahydro-6R-(L-erythro) biopterin, i.e., 5,6,7,8-tetrahydro-6R-(L-erythro-1,2-dihydroxypropyl)-pterin; 6,7-[8H]-H₂-biopterin: 6,7-dihydro-[8H]-6R-(L-erythro)-biopterin, i.e., 6,7-[8H]-dihydro-6R-(L-erythro-1',2'-dihydroxypropyl)-pterin; H₂-biopterin, 7,8-H₂-biopterin: 7,8-dihydro-6-(L-erythro)-biopterin, i.e., 7,8-dihydro-6-(L-erythro-1,2-dihydroxypropyl)-pterin; 4-amino-H₄-biopterin: 5,6,7,8-tetrahydro-2,4-diamino-6(R,S)-6-(L-erythro-1, 2-dihydroxypropyl)-pteridine; H₄-neopterin: 5,6,7,8-tetrahydro-6(R,S)-D-erythro-neopterin, i.e., 5,6,7,8-tetrahydro-6(R,S)-6-(D-erythro-1,2,3-trihydroxypropyl)-pterin; 6-methyl-H₄-pterin: 5,6,7,8-tetrahydro-6(R,S)-6-methyl-pterin; sepiapterin: 7,8-dihydro-6-lactoyl-pterin, i.e., 7,8-dihydro-6-(1-oxo-2-hydroxypropyl)-pterin. Arg: L-arginine; Asc: ascorbate; DHPR: dihydropteridine reductase; NHA: *N*-hydroxy-L-arginine; NOS: nitric oxide synthase.

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