MINIREVIEW

Tetrahydrobiopterin, Cytokines, and Nitric Oxide Synthesis[1] (44331)

ERNST R. WERNER,*,[2] GABRIELE WERNER-FELMAYER,* AND BERND MAYER†

Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Innsbruck, Austria, and Institute for Pharmacology and Toxicology,† University of Graz, Graz, Austria*

Abstract. **Nitric oxide synthases require a surprisingly rich selection of cofactors to perform the conversion of L-arginine to citrulline and nitric oxide (NO): NADPH, FAD, FMN, heme and tetrahydrobiopterin. In a previous minireview in this journal we summarized work concerning the induction of tetrahydrobiopterin biosynthesis by cytokines, which yields increased intracellular tetrahydrobiopterin concentrations supporting NO formation by intact cells (P.S.E.B.M. 203:1–12). The present review updates work on the induction of tetrahydrobiopterin biosynthesis by cytokines, and summarizes recent advances in research of tetrahydrobiopterin dependence of the NO synthase reaction. Studies using recombinant NO synthases and site-directed mutations thereof have localized several amino acids critical for tetrahydrobiopterin binding, which are discussed in reference to the recently published crystal structure of the dimer of the oxygenase domain of murine inducible NO synthase with substrate and pterin. Allosteric actions of tetrahydrobiopterin on NO synthases are stabilization of dimers, stabilization of a conformation with high-spin heme iron, and support of binding of the substrate L-arginine. Since the 4-amino analog of tetrahydrobiopterin, which is a dihydropteridine reductase inhibitor, supports these allosteric actions but inhibits the enzyme activity, tetrahydrobiopterin appears to play a redox-active role in stimulating the NO synthase reaction in addition to its allosteric actions on NO synthases. Amelioration of endothelial dysfunction by tetrahydrobiopterin in animal models and in humans *in vivo* has been observed. It remains to be investigated, however, to what extent the role of tetrahydrobiopterin as cofactor of NO synthases contributes to these *in vivo* effects of tetrahydrobiopterin.** [P.S.E.B.M. 1998, Vol 219]

As outlined in our earlier review (1), activating cytokines like γ-interferon tumor necrosis factor-α, or interleukin-1 potentiate the activity of guanosine 5′ triphosphate (GTP) cyclohydrolase I, the first and rate-limiting enzyme of tetrahydrobiopterin biosynthesis. Dihydroneopterin triphosphate, the product of the GTP cyclohydrolase I reaction, is then converted to tetrahydrobiopterin by the action of the subsequent enzymes of the pathway, 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase, which are constitutively present in most cells and only marginally regulated by cytokines. Table I gives an updated overview of cultured cells studied for the induction of tetrahydrobiopterin biosynthesis. In non-primate cells, nitric oxide (NO) synthase is induced in parallel to GTP cyclohydrolase I especially when the cells are stimulated by a mixture of cytokines. Induction of GTP cyclohydrolase I thus provides the cofactor tetrahydrobiopterin for NO synthesis. The importance of this increase in intracellular levels of tetrahydrobiopterin for NO synthesis can be demon-

Our experimental work mentioned was supported by grants from the Austrian Science Funds "zur Förderung der wissenschaftlichen Forschung", P11301 (to E.R.W.) and P11478 (to B.M.), as well as by the "Jubiläumsfonds" of the National Bank of Austria, project 5445 (to G.W.F.).

[1] This is an update of a previously published minireview entitled, "Tetrahydrobiopterin and Cytokines" PSEBM 203:1–12, 1993

[2] To whom requests for reprints should be addressed at Institut für Medizinische Chemie und Biochemie, der Universität Innsbruck, Fritz-Pregl-Str. 3, A-6020 Innsbruck, Austria. E-mail: ernst.r.werner@uibk.ac.at

0037-9727/98/2193-171$10.50/0

Table I. Induction of Pteridine Synthesis in Cultured Cells by Cytokines[a]

Cell	Stimuli		Reference
	positive	negative	
Human			
A431 epidermoid carcinoma	IFNγ		(108)
A498 kidney carcinoma	IFNγ		(108)
A549 lung carcinoma	IFNγ		(108)
B lymphocytes (blood)	IFNγ, IL-2, PWM		(109)
Endothelial cells, umbilical vein	IFNγ, TNFα, LPS		(32, 110, 111)
Endothelial cells ea.hy926	IFNγ, TNFα, IL-1, LPS;	IL-4, IL-10, TGFβ	(112)
Fibroblasts (dermis)	IFNγ, TNFα, IL-1		(1, 108, 113)
HUT102 T lymphocytes	IFNγ, IL-2		(114, 115)
Macrophages, peripheral blood	IFNα,β,γ, TNFα, LPS		(116–119)
Mononuclear cells, periph. blood	IFNα,β,γ, IL-2, LPS, PHA, PWM		(114–116, 118, 119–123)
ME-180 cervial carcinoma cells	IFNγ		(124)
MT-2 T cells (umbilical cord)	IFNγ, IL-2		(30, 114)
SK-HEP-1 hepatoma	IFNγ		(108)
Smooth muscle cells	IFNγ, TNFα, IL-1, LPS		(125)
T lymphocytes (periph. blood)	PHA		(30, 114)
Thyrocytes	IL-1α, IFN-γ		(126)
THP-1 monocytoma	IFNα,β,γ, TNFα, LPS		(127)
T24 bladder carcinoma	IFNγ		(108, 113)
U138MG glioblastoma	IFNγ		(108)
U373 glioblastoma	IFNγ		(128)
U937 monocytoma	IFNγ, PHA		(30)
Mouse			
Fibroblasts, dermal	IFNγ, TNFα		(129)
J774A1 macrophage line	constitutive	CsA, FK506	(129, 130)
L929 fibroblasts	IFNγ, TNFα		Werner-Felmayer *et al.*, unpublished
Macrophages, B10-BR, resident	LPS		(131)
Macrophages, BALB/c, resident	IFNγ, TNFα		(128)
Macrophages, C3H/HeN, elicited	LPS	dexamethasone	(112)
N1E-115	LPS		(132)
OVA T-cell line	IL-2		(131)
P388D1 macrophage line	constitutive		(129)
Raw 264 macrophage line	constitutive, IFNγ, LPS		(49, 50, 133)
Rat			
C6 glioma	LPS, TNFα		(134)
Cardiac endothelial cells	IL-1, IFNγ,	glucocorticoids	(8)
Glial cells	LPS, IFNγ, TNFα, IL-2		(128)
Granulosa cells	FSH, gonadotropin		(135)
Hepatocytes	LPS + TNFα + IL-1 + IFNγ		(136)
Macrophages elicited	IFNγ + LPS		(133)
Mesangial cells	IL-1, cAMP	dex, ET-1	(10, 137–139)
PC-12 pheochromocytoma	EGF, NGF, dex		(36, 140)
Smooth muscle cells	IFNγ, IL-1, LPS, TNFα, LTA	TGFβ, thrombin	(5, 21, 31, 141–143)
Superior cervical ganglia		LIF, CNTF	(144)

[a] Abbreviations: CNTF, ciliary neurotrophic factor; DAHP, 2,4-diamino 6-hydroxy pyrimidine; dex, dexamethasone; ET, endothelin; FSH, follicle stimulating hormone; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NGF, nerve growth factor; PHA, phytohemagglutinin; PWM, poke weed mitogen; TNF, tumor necrosis factor.

strated by using inhibitors of tetrahydrobiopterin biosynthesis. Depletion of intracellular tetrahydrobiopterin levels leads to a drop in NO formation by both inducible NO synthase (iNOS; measured as nitrite plus nitrate in culture supernatants) and constitutive NO synthases (eNOS; nNOS; measured as cGMP accumulation following a Ca^{2+} trigger). Table II gives an updated summary of cells in which a drop in NO formation by depletion of intracellular tetrahydrobiopterin has been observed. Many of these investigations have used the protocol of our initial study (2) to inhibit intracellular tetrahydrobiopterin biosynthesis: 2,4-diamino 6-hydroxy pyrimidine (DAHP) is used to inhibit GTP cyclohydrolase I (3), and intracellular tetrahydrobiopterin levels can be restored in the presence of this inhibitor by se-

Table II. Inhibition of NO Formation in Cultured Cells by Depletion of Intracellular Tetrahydrobiopterin[a]

Cell	NOS	Inhibitor	Reference
Chicken macrophages	iNOS	DAHP	(145, 146)
Murine dermal fibroblasts	iNOS	DAHP	(2)
Murine brain endothelial cells	iNOS	DAHP, NAS	(147)
Murine vascular endothelial, send	iNOS	PCC, dicumarol	(7)
Murine macrophages	iNOS	DAHP, PCC, NAS, dex	(6, 133)
Murine proximal tubule cells	iNOS	DAHP	(148)
Human endothelial cells, umbilical vein	eNOS	DAHP	(110, 111)
Human cervical carcinoma ME-180	nNOS	DAHP	(124)
Human thyrocytes	iNOS	DAHP	(126)
Porcine endothelial cells, aortic	eNOS	DAHP	(149)
Rat cardiac endothelial cells	iNOS	glucocorticoids	(8)
Rat cardiac myocytes	iNOS	DAHP	(20)
Rat glial cells	iNOS	DAHP	(128, 150)
Rat glomerular mesangial cells	iNOS	DAHP	(138)
Rat hepatocytes	iNOS	DAHP	(137)
Rat lung fibroblasts	iNOS	DAHP	(151)
Rat smooth muscle cells, aortic	iNOS	DAHP, NAS	(5, 21, 152)

[a] Abbreviations: DAHP, 2,4-diamino 6-hydroxypyrimidine; NAS, N-acetyl serotonin; PCC, phenprocoumon.

piapterin. Conversion of sepiapterin to tetrahydrobiopterin can be blocked by methotrexate (4). Some studies also used sepiapterin reductase inhibitors to deplete intracellular tetrahydrobiopterin (5–7) or glucocorticoids like dexamethasone (6, 8). In addition to its effects on transcription of the iNOS gene, dexamethasone reduces iNOS mRNA stability and translation and increases the degradation of the iNOS protein (9). Moreover, dexamethasone reduces induction of tetrahydrobiopterin biosynthesis (6, 8, 10, 11) and inhibits arginine transport (8), two mechanisms further downregulating the NO synthesis of dexamethasone-treated cells.

The fact that modulation of intracellular tetrahydrobiopterin levels alters the amount of NO formed by intact cells seems to be surprising in view of the observation that tetrahydrobiopterin binds tightly enough to NO synthase to remain bound upon purification of the enzyme (12, 13). Experiments with radiolabeled tetrahydrobiopterin, however, explained this paradox. As shown for nNOS (14) and iNOS (15), NO synthase contains two identical, highly anticooperative binding sites for tetrahydrobiopterin. Occupation of the first, high-affinity site decreases the affinity of the NO synthase dimer for the second tetrahydrobiopterin molecule by almost three orders of magnitude. Manipulation of tetrahydrobiopterin levels in intact cells thus may not alter occupation of the first, high-affinity site but change the degree of the occupation of the low affinity binding site and thereby modulate enzyme activity.

DAHP is not a very potent inhibitor of GTP cyclohydrolase I, and thus must be used in millimolar concentrations to exert its effects. This can cause additional actions on cells, (e.g., an attenuation of the induction of NO synthase in cases of suboptimal stimulation by a single stimulus in freshly isolated murine macrophages (16) or in human mesangial cells (17). In vascular smooth muscle cells, tetrahydrobiopterin was reported to post-transcriptionally stabilize iNOS mRNA (18). Studies with human mesangial cells showed that the effect of tetrahydrobiopterin on expression is not specific for iNOS mRNA, but is also observed with another cytokine-induced enzyme, cyclooxygenase 2 (19). However, using murine fibroblasts and optimal stimulation with combined stimuli, these side effects of DAHP are not observed, and a depletion of intracellular tetrahydrobiopterin is seen without changes in iNOS and GTP cyclohydrolase I mRNA and protein levels (Fig. 1). In accordance with our findings, iNOS mRNA expression in IL-1-treated

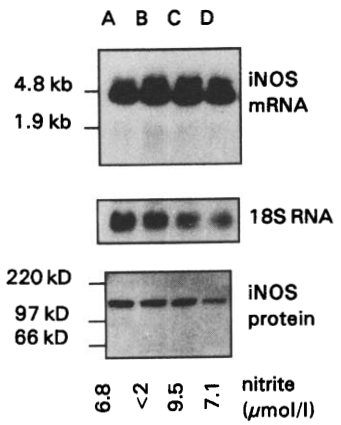

Figure 1. Independence of expression of iNOS mRNA and iNOS protein from treatment of cells by 2,4-diamino-6-hydroxy pyrimidin (DAHP). Murine fibroblasts were cultivated, stimulated with a combination of γ-interferon and tumour necrosis factor-α as described in Ref. 2, and treated for 24 hr with (A) no further treatment; (B) 5 mM DAHP; (C) 100 μ*M* sepiapterin; (D) 5 m*M* DAHP + 100 μ*M* sepiapterin. Cells were then collected and nitrite determined in supernatants by the Griess reaction. Northern blots were performed using 10 μg of total RNA and a ^{32}P-labeled murine iNOS-cDNA-probe. Western blots were performed by standard techniques using 50 μg of cellular protein, and the iNOS band visualized with a rabbit antiserum to murine holo-iNOS, an anti-rabbit IgG horseradish peroxidase conjugate and the ECL detection system (Amersham, Little Chalfont, U.K.). Murine iNOS cDNA and antiserum were kind gifts of Qiao-wen Xie and Carl Nathan (Cornell University Medical College, New York, NY).

cardiac myocytes was not altered by addition of DAHP (20). On the other hand, side effects of N-acetyl serotonin on expression of iNOS have been observed (21). Thus, depending on the particular conditions and cells used, DAHP may have actions in addition to tetrahydrobiopterin depletion, and modulation of intracellular tetrahydrobiopterin may have more general actions on cells in addition to influencing NO synthase activity. However, as outlined in the example in Figure 1, these additional effects do not necessarily occur in all cases, and DAHP and sepiapterin are able to modulate NO synthase activity in intact cells without overt side effects on iNOS mRNA and protein levels.

Interestingly, coordinate regulation of tetrahydrobiopterin biosynthesis by cytokines has not only been observed for NO synthase. In PC-12 rat pheochromocytoma cells, for example, NGF and EGF upregulate GTP cyclohydrolase I and tyrosine hydroxylase, a tetrahydrobiopterin-dependent enzyme, in a coordinate way (22). In hepatocytes, where both phenylalanine hydroxylase and iNOS are present, there is no limitation of either activity by competition for the common cofactor, tetrahydrobiopterin (23). Only phenylalanine but not arginine increases biopterin synthesis in the liver by action of the recently cloned GTP cyclohydrolase I feedback regulatory protein (24, 25). GTP cyclohydrolase I was found to colocalize with tyrosine hydroxylase but not with NO synthase in rat brain (26). This is in line with the orders of magnitude higher concentrations of tetrahydrobiopterin required for stimulation of aromatic amino acid hydroxylases as compared to NO synthases (see below). On the other hand, hypoxia caused a parallel induction of nNOS and GTP cyclohydrolase I mRNAs in rat cerebella (27).

Although the human (28, 29) and murine gene (28) for GTP cyclohydrolase I have been cloned, the molecular mechanism of stimulation of GTP cyclohydrolase I by cytokines is not yet known. Cytokines cause an increased level of GTP cyclohydrolase I mRNA (30, 31), which is a result of increased transcription as demonstrated by the nuclear run on assays (32). The increase in GTP cyclohydrolase I mRNA is inhibited by pyrrolidine dithiocarbamate (33), indicating a possible involvement of nuclear factor kappa B in the induction. Although in this case iNOS induction is affected in parallel, it is clear from experiments with single stimuli that single cytokines can stimulate either induction of NO synthase or GTP cyclohydrolase I alone. Thus the signal transduction events leading to induction of GTP cyclohydrolase I may be similar in some steps but cannot be identical to those leading to NO synthase expression. In γ-interferon treated THP-1 cells, we recently characterized GTP cyclohydrolase I mRNAs. We found two species: one 2.9-kB mRNA, which with the exception of two nucleotides in the untranslated region is identical to the human pheochromocytoma GTP cyclohydrolase I mRNA (34), as well as a new splicing variant encoding a protein with an altered carboxy terminus. No indication of a cytokine-induced form of GTP cyclohydrolase I arising from a gene different from the gene encoding the constitutive liver enzyme was detected (G. Werner-Felmayer *et al.*, unpublished data).

Induction of tetrahydrobiopterin biosynthesis by cytokines and attenuation of NO synthesis by inhibition of tetrahydrobiopterin biosynthesis have also been observed *in vivo*. The first studies on GTP cyclohydrolase I induction *in vivo* were not done with cytokines, but were performed using insuline and reserpine in rats, which led to an increase in GTP cyclohydrolase I activity in the adrenal medulla (35). As was shown more recently, cortisol also increases GTP cyclohydrolase I mRNA in rat adrenal medulla (36). Induction of GTP cyclohydrolase I in humans can be conveniently measured by determining neopterin concentrations in body fluids (reviewed in Ref. 37). Increased body fluid concentrations of nitrite plus nitrate parallel the increase in neopterin in humans (e.g., in bone marrow transplantation (38)), and in HIV infection (39, 40). A model of experimental septic shock in baboons confirmed the parallel induction of both pathways, pteridine and NO synthesis, under immunostimulant but not under hemorrhagic conditions (41). *In vivo* studies with rats demonstrated the induction of GTP cyclohydrolase I activity (42) and mRNA (31) following lipopolysaccharide (LPS) treatment, as well as the ability of DAHP (43) and N-acetyl serotonin (21) to attenuate NO production. A study focusing on the heart of LPS-treated rats confirmed that induction of GTP cyclohydrolase I and NO synthase also occurs in this tissue (44); parallel induction is also seen in ventilatory and limb muscles (45). Increases in plasma biopterin and nitrite plus nitrate levels differ markedly between mammalian species (e.g., between rat and rabbit (46)). In human primordial placenta homogenates, Ca^{2+}-dependent NO synthesis can be markedly stimulated by addition of tetrahydrobiopterin (47). Finally, it is of interest that parallel synthesis of tetrahydrobiopterin and nitric oxide is not restricted to mammals, but occurs also in lower eukaryotes (e.g. in the slime mold *Physarum polycephalum* (48)).

Role of Tetrahydrobiopterin in the NO Synthase Reaction

Since the initial findings of stimulation of NO synthase activity by tetrahydrobiopterin (49–51) there has been a lively debate about the role of tetrahydrobiopterin in the NO synthase reaction. From the beginning it was evident that tetrahydrobiopterin stimulation of NO synthase shows features different from stimulation of phenylalanine hydroxylase by tetrahydrobiopterin (52). Much lower (submicromolar) concentrations of tetrahydrobiopterin are required by NO synthase to yield half maximal stimulation. NO synthase shows a much higher selectivity than phenylalanine hydroxylase (53) for the 6R-L-erythro-1,2-dihydroxylpropyl side chain (i.e., the side chain occurring in the natural tetrahydrobiopterin cofactor). Both functional (50) and radioligand binding data (54, 55) demonstrate that tetrahydrobiopterin binds to NO synthase with much higher affinity than other pterins. In NO synthase, the stoichiometry of the

formed product to added tetrahydrobiopterin is not 1:1 as in phenylalanine hydroxylase but 10:1 (12, 56). No overall reaction occurs in the absence of NADPH with added tetrahydrobiopterin, and a (variable) basal activity is observed in the absence of added tetrahydrobiopterin due to tight binding of the cofactor (12, 13). Since active NO synthase dimers contain two heme groups that are essential for catalysis (57), the question arises as to why tetrahydrobiopterin is needed in the NO synthase reaction (58). The presence of the heme together with FAD and FMN seems to make the redox properties of tetrahydrobiopterin not necessarily required to formulate a reaction mechanism. Indeed, allosteric actions of tetrahydrobiopterin on NO synthase are the most unambiguously defined roles of the cofactor known at present. Tetrahydrobiopterin stabilizes the dimeric, active state of the enzyme (56, 57, 59, 60). The availability of heme appears to be the most critical parameter for correct assembly of the active dimer (61, 62) and formation of a high-affinity binding site for tetrahydrobiopterin (63). The reported, tetrahydrobiopterin-triggered conversion of the inactive low-spin to the active high-spin conformation of the enzyme (64–66) is caused by the action of an equilibrium (14). The conversion of the low-spin to the high-spin form also slowly occurs in the absence of tetrahydrobiopterin. Since tetrahydrobiopterin binds only to the high-spin, but not to the low-spin state, addition of tetrahydrobiopterin shifts the equilibrium toward the high-spin state. The nature of the conformational change in NO synthase caused by tetrahydrobiopterin and its role in dimerization have become clearer by a comparison of the crystal structures of N-terminally truncated murine iNOS oxygenase domains with (67) and without (68) tetrahydrobiopterin. Upon binding of the pterin, drastic changes occur in the structure causing mobile, hydrophobic residues that are exposed in the monomer (68) to refold and contribute to formation of a substrate channel and the pterin binding sites (67). The dimer interface is composed of more than 85 residues per subunit (67), explaining the extraordinarily high stability of NOS dimers (60). Refolding of nNOS and iNOS upon binding of tetrahydrobiopterin restricts access of ligands to the distal heme pocket, so that only very small ligands like CO, NO, or O_2 can reach the heme (69).

Consistent with the role of tetrahydrobiopterin in stabilization of the active conformation of the enzyme, overexpressed NO synthase requires an adequate supply of tetrahydrobiopterin to become active (66, 70–74). Binding of tetrahydrobiopterin to nNOS shows a positive cooperativity with arginine, and arginine binding in turn is enhanced by tetrahydrobiopterin (54). Consistent with this finding, an altered K_m of NOS for L-arginine has been observed in a mouse strain with tetrahydrobiopterin deficiency (75). A detailed comparison of tetrahydrobiopterin-containing and tetrahydrobiopterin-free nNOS showed two identical highly anticooperative binding sites for tetrahydrobiopterin (14).

In addition to these well-defined allosteric roles of tetrahydrobiopterin as a redox-active cofactor has been researched. In particular, it was tested whether or not a redox cycle similar to that seen in phenylalanine hydroxylation occurs in NO synthases (Fig. 2). Following hydroxylation of the substrate phenylalanine, a 4a hydroxy pterin derivative is formed, which after dehydratation by carbinolamine dehydratase to quinonoid 6,7 [8H] dihydrobiopterin is then recycled by dihydropteridine reductase to the active 5,6,7,8 tetrahydro derivative. Rat nNOS did not show a dihydropteridine reductase activity in a first investigation (56). Only when quinonoid 6,7 [8H] dihydrobiopterin was added in much higher concentrations than required for maximal stimulation of the overall NO synthase reaction, was a reduction of quinonoid 6,7[8H] dihydrobiopterin observed at a rate comparable to product formation (76). Using high concentrations of enzyme, minor amounts of N-hydroxyarginine, an intermediate of the NO synthase reaction (77), are formed in the absence of NADPH in dependence of added tetrahydrobiopterin (78). A comparison of the action of 6-methyl tetrahydropterin and its 5 deaza analog, which cannot undergo the redox cycle outlined in Figure 2, also indicated that redox chemistry might be involved. Although 6-methyl tetrahydropterin is only a poor stimulator of iNOS, a clear difference from the 5-deaza compound was observed, which even inhibited the small stimulatory effect of 6-methyl tetrahydropterin (54). However, it is not clear to what extent these two compounds support the allosteric action of tetrahydrobiopterin on NO synthases described above. Support of a redox contribution by the pterin comes from investigations using the 4-amino analog of tetrahydrobiopterin. This compound is an inhibitor of dihydropteridine reductase, and, unlike methotrexate, strongly binds to NO synthase. This binding inhibits stimulation of NO synthase by tetrahydrobiopterin with high efficiency (55). Since the 4-amino analog of tetrahydrobiopterin also exerts the allosteric actions on NO synthase in a manner comparable to tetrahydrobiopterin (15, 79), it seems clear that the allosteric actions of tetrahydrobiopterin on NO synthase cannot fully explain its stimulating role in the reaction. A similar conclusion can be drawn from a comparison of several di- and tetrahydropterins on features of the NO synthase thought to require pterin (80). Whereas NO synthesis and the ability to increase heme-dependent NADPH oxidation was supported only by tetra-, but not by dihydropterins, other features such as subunit assembly into a dimer were also promoted by dihydropterins (80). In the crystal structure of a truncated iNOS oxygenase domain, tetrahydrobiopterin is located at some distance to the guanidino group of L-arginine and close to the heme. This renders a role in direct hydroxylation of the substrate unlikely and indicates the possibility of electronic influences on the heme-oxygen complex (67). Indeed, tetrahydrobiopterin accelerated the decay of ferrous oxygen complex of the nNOS oxygenase domain by a factor of 70, whereas L-arginine stabilized this complex (81). Studies of the reaction of nNOS with oxygen at low tem-

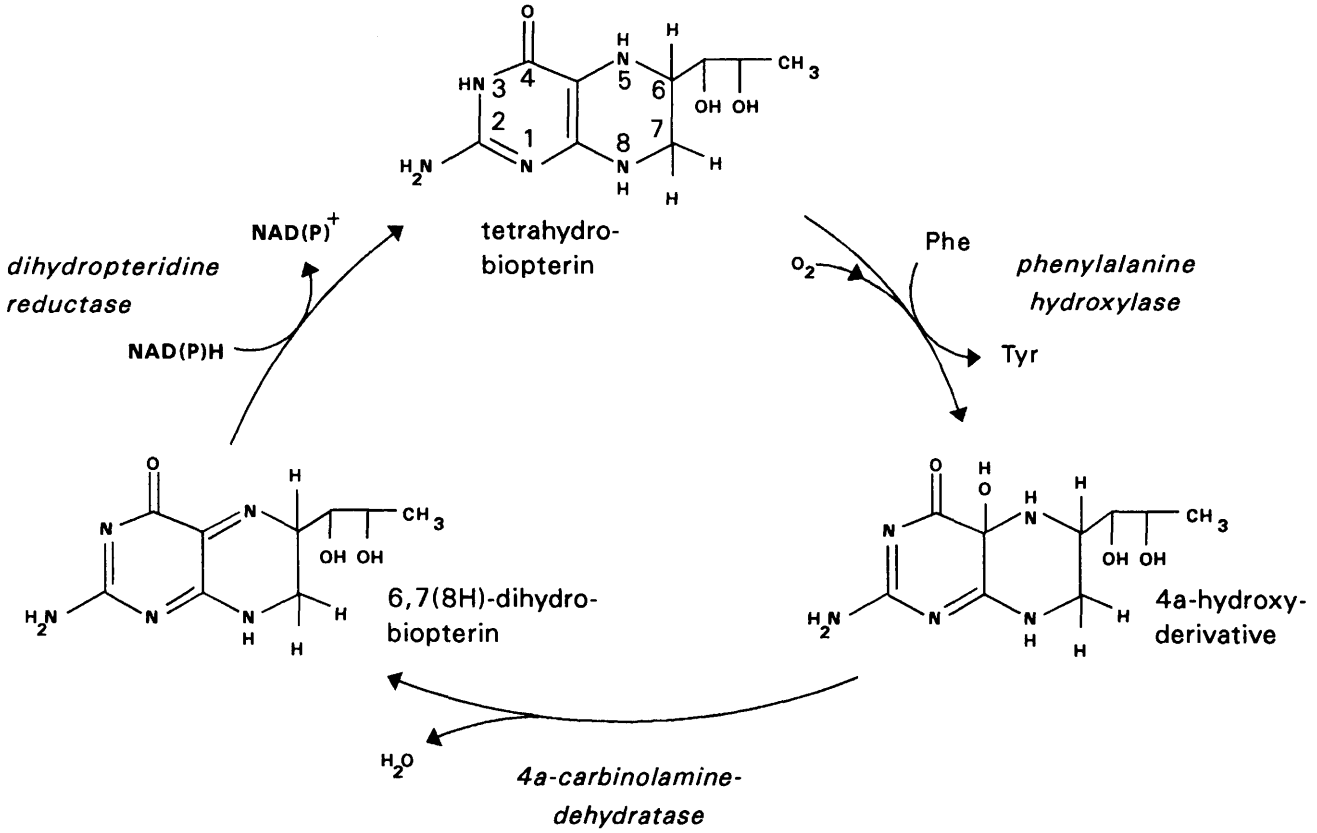

Figure 2. Recycling of tetrahydropteridines during stimulation of the phenylalanine hydroxylase reaction.

peratures suggested that tetrahydrobiopterin plays a role in reductive activation of the oxy-ferrous complex (82).

Tetrahydrobiopterin has been shown to attenuate the inhibition of NO synthase by its product, NO (83). This effect is most likely due to a removal of NO by reaction with superoxide formed in the course of the autooxidation of tetrahydrobiopterin (84). In addition, allosteric actions of tetrahydrobiopterin on the iNOS oxygenase domain destabilize the heme-NO complex (85), an effect that may also contribute to preventing NO synthase autoinactivation.

Mutational Studies to Localize a Tetrahydrobiopterin Binding Module in NO Synthases

Site-directed mutagenesis, deletion of parts of the protein and expression of parts of the protein ("modules"), has been used to localize residues important for tetrahydrobiopterin binding (Table III). Analysis of partially purified NO synthase for tetrahydrobiopterin content by HPLC (86, 87), binding of ^{3}H-labeled tetrahydrobiopterin (88), or influence of tetrahydrobiopterin on binding of radiolabeled N^{G}-nitro-L-arginine were used to check for tetrahydrobiopterin interaction with NOS. Mutations G450A and A453I in murine iNOS abolished binding of tetrahydrobiopterin (86). Mutation of the corresponding residues in bovine eNOS (G442A, A445I) severely inhibited dimer formation of eNOS as shown with the yeast two-hybrid system (89). However, this dimerization is not dependent on tetrahydrobiopterin, indicating that not only the tetrahydrobiopterin binding site may be altered but the whole structure of the protein. The recently published crystal structure of truncated iNOS oxygenase domain with bound pterin (67) nicely demonstrates how the two regions in the enzyme indicated to be important for pterin binding by the mutagenesis studies (Table III) interact with the pterin. It is bound by residues 108–114 (N-terminal binding domain) of one subunit and the so-called "helical lariat" (residues 453–476) of the other subunit (67), where the previously identified homology to other pterin-dependent enzymes is located (86).

Influence of Tetrahydropterins on Endothelial Function

Since tetrahydrobiopterin had been demonstrated to support NO synthesis in intact cells, investigations have been carried out to test the effects of tetrahydrobiopterin on endothelial function in isolated arteries as well as in animals and humans. Van Amsterdam and Wemer (90) first demonstrated a vasodilatation mediated by tetrahydrobiopterin in precontracted rat aortae, that were antagonized by NOS inhibition. Similarly, sepiapterin, which is converted to tetrahydrobiopterin by the action of sepiapterin reductase and dihydrofolate reductase, caused dilatation of canine cerebral arteries. This vasodilatation was only seen in the presence of intact endothelium and was almost abolished by the use of

Table III. Effect of Mutations of NOS Isoforms on Tetrahydrobiopterin Effects to NOS[a]

Residue/mutation	Position			Relevant for H_4biopterin binding	References
	nNOS	iNOS	eNOS		
deletion		1–58(hu)		no	(153)
deletion		1–65(mu)		no	(88)
Cys → Ala			99(hu)	yes	(154)
Cys → Ala		109(mu)		yes	(88)
deletion		1–114(mu)		yes	(88)
deletion		1–117(mu)		yes	(88)
Cys → Ala		200(hu)		yes	(153)
Cys → Ala		217(hu)		no	(153)
Cys → Ala		228(hu)		no	(153)
Cys → Ala		290(hu)		no	(153)
Cys → Ala		384(hu)		no	(153)
Ala → Ile		447(mu)		no	(86)
Gly → Ala		450(mu)		yes	(86)
Cys → Ala		451(mu)		no	(86)
Cys → Ala		457(hu)		no	(153)
Pro → Ala		452(mu)		no	(86)
Ala → Ile		453(mu)		yes	(86)
Pro → Ala		461(mu)		no	(86)

[a] Abbreviations: H_4biopterin, 6R-5,6,7,8-tetrahydro-L-*erythro*-biopterin; hu, human; mu, murine.

Ca^{2+}-free medium (91). Analogous observations were made using tetrahydrobiopterin in mouse pial arterioles (92), and with sepiapterin and 6-methyl tetrahydropterin in pig arterioles after ischemia/reperfusion injury (93). When canine arteries were treated with DAHP to deplete tetrahydrobiopterin, Ca^{2+} agonist–mediated dilatations, which are inhibited by NOS inhibitors, became catalase sensitive (94). Catalase had no effect both on dilatations of vessels in the absence of DAHP and on arteries treated with DAHP plus 6-methyl tetrahydropterin (94). Cosentino and Katusic (94) interpreted their finding as formation of hydrogen peroxide by pterin-deficient NOS. Tetrahydrobiopterin-deficient nNOS had previously been shown to produce hydrogen peroxide even in the presence of saturating levels of arginine due to the uncoupled reduction of oxygen by NADPH (95). However, in contrast to nNOS, recombinant G2A eNOS did not show a comparable increase in uncoupled NADPH oxidation when tetrahydrobiopterin was omitted (96). These findings are in contrast to results presented by Wever *et al.* (97), who measured eNOS-catalyzed superoxide formation by lucigenin-enhanced chemiluminescence. This discrepancy is explained by the fact that reduction of lucigenin by NADPH-dependent reductases (including eNOS) results in an artificial generation of superoxide due to reduction of molecular oxygen by the reduced lucigenin species thus formed (98, 99). Therefore, lucigenin cannot be used as a probe to detect superoxide production in eNOS or other biological systems.

6-Methyl tetrahydropterin is only a poor cofactor of NOS, and a more than 100-fold higher concentration of this compound as compared to the natural cofactor tetrahydrobiopterin is required to restore DAHP-inhibited cGMP response to Ca^{2+} ionophore in cultured porcine endothelial cells (K. Schmidt, personal communication). Nevertheless, 6-methyl tetrahydropterin is able to correct diabetes-induced endothelial dysfunction in rats when added to isolated aortic rings in high concentrations (100 μM). In humans, tetrahydrobiopterin restores endothelial function in hypercholesterolemia while exhibiting little effect in controls (100). These results have prompted the speculation that diabetes or hypercholesterolemia is associated with tetrahydrobiopterin deficiency. Unfortunately, no experimental evidence supporting this assumption has been presented thus far.

Possible contributions of tetrahydrobiopterin action on blood vessels are shown in Figure 3. Tetrahydrobiopterin stimulates NO synthase, and, as has been observed with nNOS but only to a much smaller extent with eNOS, reduces uncoupled formation of superoxide and hydrogen peroxide by the enzyme. In addition, tetrahydrobiopterin chemically reduces oxygen to superoxide, which rapidly reacts with NO to form peroxynitrite. Therefore, pure nNOS forms peroxynitrite rather than nitric oxide unless incubated in the presence of high amounts of superoxide dismutase (84). Superoxide on its own is a vasoconstrictor (101). On the other hand, hydrogen peroxide formed from superoxide by superoxide dismutase, causes dilatations. Intriguingly, these dilatations by hydrogen peroxide in turn appear to be mediated by NO (102). And, finally, tetrahydrobiopterin was shown to act on N-type Ca^{2+} channels independently of its NOS stimulatory capacity, as has been shown by investigations into the mechanism of dopamine release in the brain, a process caused by tetrahydrobiopterin (103). Whether or not a similar mechanism is operative in blood vessels has yet to be investigated.

Which of the contributions shown in Figure 3 mediates pterin-dependent vasodilations, or pterin-dependent corrections of endothelial dysfunction *in vivo*? This cannot be

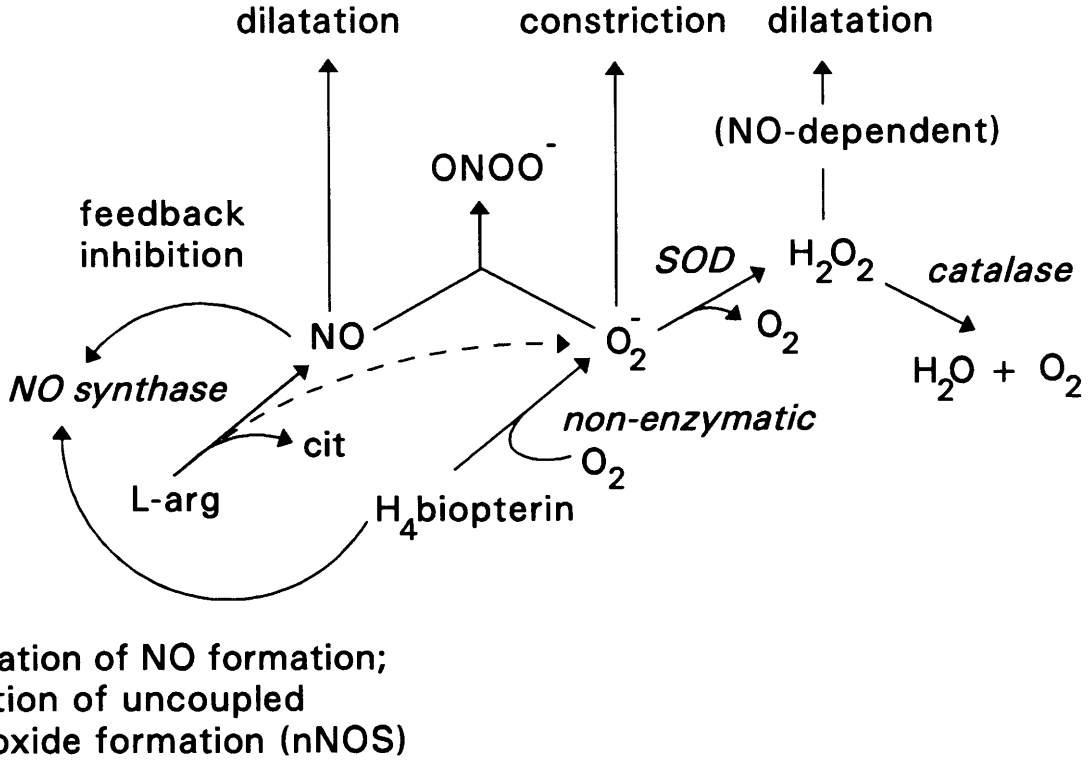

Figure 3. Possible enzymatic and nonenzymatic reactions of added tetrahydrobiopterin leading to vasodilatation or vasoconstriction.

decided unequivocally by the studies performed thus far. A study comparing the dose-response of the natural cofactor, tetrahydrobiopterin, with a compound of similar chemical properties but much lower affinity to NO synthase, could decide between the enzymatic and chemical effects shown in Figure 3. The reported effects of 6-methyl tetrahydropterin, a poor stimulator of NO synthase, might point to a considerable contribution of chemical effects. On the other hand, these poor stimulatory properties might be counteracted by favorable pharmacokinetic properties, rendering the concentrations reached *in vivo* to be high enough to exert direct effects on NO synthase. Another difficulty with the current interpretations published is the very low amount of uncoupled NADPH reduction by recombinant eNOS (in contrast to nNOS) when tetrahydrobiopterin is omitted from the reaction mixture (96). In the recombinant eNOS and nNOS enzymes, N^G-methyl-L-arginine has almost no effect on uncoupled NADPH oxidation, whereas N^G-nitro-L-arginine is a potent inhibitor (96). In arteries from spontaneously hypertensive rats, however, N^G-methyl-L-arginine was much more efficient in inhibiting superoxide release than N^G-nitro-L-arginine methyl ester (104). These discrepancies suggest that the effects observed in tissues cannot be explained directly by data obtained from experiments with purified NOS enzymes. Intriguingly, reduced compounds other than tetrahydrobiopterin like N5-methyl tetrahydrofolate (105) or ascorbate (106) are also able to correct impaired endothelial function, and this effect could be inhibited by N^G-methyl-L-arginine as well (106). Thus, the mechanisms accounting for the effects of tetrahydrobiopterin observed in humans *in vivo* and in animal models might be much more complicated than discussed thus far. The action of different preparations of low-density lipoproteins on NOS-mediated generation of superoxide in endothelial cells (107) may serve as an example of this complexity. It will be interesting to learn whether or not the effects of tetrahydrobiopterin depletion and replenishment are connected to these actions of low-density lipoproteins.

1. Werner ER, Werner Felmayer G, Wachter H. Tetrahydrobiopterin and cytokines. Proc Soc Exp Biol Med **203:**1–12, 1993.
2. Werner Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter H. Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. J Exp Med **172:**1599–1607, 1990.
3. Gal EM, Nelson JM, Sherman AD. Biopterin III: Purification and characterization of enzymes involved in cerebral synthesis of 7,8-dihydrobiopterin. Neurochem Res **3:**69–88, 1978.
4. Nichol CA, Smith GK, Duch DS. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. Annu Rev Biochem **54:**729–764, 1985.
5. Gross SS, Levi R. Tetrahydrobiopterin synthesis: An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. J Biol Chem **267:**25722–25729, 1992.
6. Schoedon G, Schneemann M, Hofer S, Guerrero L, Blau N, Schaffner A. Regulation of the L-arginine-dependent and tetrahydrobiopterin-dependent biosynthesis of nitric oxide in murine macrophages. Eur J Biochem **213:**833–839, 1993.
7. Schoedon G, Blau N, Schneemann M, Flury G, Schaffner A. Nitric oxide production depends on preceding tetrahydrobiopterin synthesis by endothelial cells: Selective suppression of induced nitric oxide production by sepiapterin reductase inhibitors. Biochem Biophys Res Commun **199:**504–510, 1994.
8. Simmons WW, Ungureanu Longrois D, Smith GK, Smith TW, Kelly RA. Glucocorticoids regulate inducible nitric oxide synthase by in-

hibiting tetrahydrobiopterin synthesis and L-arginine transport. J Biol Chem **271:**23928–23937, 1996.
9. Walker G, Pfeilschifter J, Kunz D. Mechanisms of suppression of inducible nitric-oxide synthase (iNOS) expression in interferon (IFN)-γ-stimulated RAW 264.7 cells by dexamethasone: Evidence for glucocorticoid-induced degradation of iNOS protein by calpain as a key step in post-transcriptional regulation. J Biol Chem **272:**16679–16687, 1997.
10. Plüss C, Werner ER, Wachter H, Pfeilschifter J. Dexamethasone differentially affects interleukin 1β- and cAMP-triggered expression of GTP cyclohydrolase I in rat renal mesangial cells. Br J Pharmacol **122:**534–538, 1997.
11. Hattori Y, Akimoto K, Nakanishi N, Kasai K. Glucocorticoid regulation of nitric oxide and tetrahydrobiopterin in a rat model of septic shock. Biochem Biophys Res Commun **240:**298–303, 1997.
12. Mayer B, John M, Heinzel B, Werner ER, Wachter H, Schultz G, Bohme E. Brain nitric oxide synthase is a biopterin- and flavin-containing multifunctional oxido-reductase. FEBS Lett **288:**187–191, 1991.
13. Hevel JM, Marletta MA. Macrophage nitric oxide synthase. Relationship between enzyme-bound tetrahydrobiopterin and synthase activity. Biochemistry **31:**7160–7165, 1992.
14. Gorren AC, List BM, Schrammel A, Pitters E, Hemmens B, Werner ER, Schmidt K, Mayer B. Tetrahydrobiopterin-free neuronal nitric oxide synthase: Evidence for two identical highly anticooperative pteridine binding sites. Biochemistry **35:**16735–16745, 1996.
15. Mayer B, Wu C, Gorren ACF, Pfeiffer S, Schmidt K, Clark P, Stuehr DJ, Werner ER. Tetrahydrobiopterin binding to macrophage inducible nitric oxide synthase expressed in *Echerichia coli:* Heme spin shift and dimer stabilization by the potent pterin antagonist 4-amino tetrahydrobiopterin. Biochemistry **36:**8422–8427, 1997.
16. Bogdan C, Werner E, Stenger S, Wachter H, Rollinghoff M, Werner Felmayer G. 2,4-Diamino-6-hydroxypyrimidine, an inhibitor of tetrahydrobiopterin synthesis, downregulates the expression of iNOS protein and mRNA in primary murine macrophages. FEBS Lett **363:**69–74, 1995.
17. Saura M, Perez Sala D, Canada FJ, Lamas S. Role of tetrahydrobiopterin availability in the regulation of nitric oxide synthase expression in human mesangial cells. J Biol Chem **271:**14290–14295, 1996.
18. Linscheid P, Schaffner A, Schoedon G. Modulation of inducible nitric oxide synthase mRNA stability by tetrahydrobiopterin in vascular smooth muscle cells. Biochem Biophys Res Commun **243:**137–141, 1998.
19. Perezsala D, Diazcazorla M, Ros J, Jimenez W, Lamas S. Tetrahydrobiopterin modulates cyclooxygenase-2 expression in human mesangial cells. Biochem Biophys Res Commun **241:**7–12, 1997.
20. Oddis CV, Finkel MS. NF-κ B and GTP cyclohydrolase regulate cytokine-induced nitric oxide production by cardiac myocytes. Am J Physiol **270:**H1864–H1868, 1996.
21. Klemm P, Hecker M, Stockhausen H, Wu CC, Thiemermann C. Inhibition by N-acetyl-5-hydroxytryptamine of nitric oxide synthase expression in cultured cells and in the anaesthetized rat. Br J Pharmacol **115:**1175–1181, 1995.
22. Anastasiadis PZ, States JC, Kuhn DM, Levine RA. Co-induction of tetrahydrobiopterin (BH4) levels and tyrosine hydroxylase activity in cultured PC12 cells. Adv Exp Med Biol **338:**227–230, 1993.
23. Pastor CM, Williams D, Yoneyama T, Hatakeyama K, Singleton S, Naylor E, Billiar TR. Competition for tetrahydrobiopterin between phenylalanine hydroxylase and nitric oxide synthase in rat liver. J Biol Chem **271:**24534–24538, 1996.
24. Milstien S, Jaffe H, Kowlessur D, Bonner TI. Purification and cloning of the GTP cyclohydrolase I feedback regulatory protein, GFRP. J Biol Chem **271:**19743–19751, 1996.
25. Yoneyama T, Brewer JM, Hatakeyama K. GTP cyclohydrolase I feedback regulatory protein is a pentamer of identical subunits: Purification, cDNA cloning, and bacterial expression. J Biol Chem **272:**9690–9696, 1997.
26. Hwang OY, Baker H, Gross S, Joh TH. Localization of GTP cyclohydrolase in monoaminergic but not nitric oxide-producing cells. Synapse **28:**140–153, 1998.
27. Guo Y, Ward ME, Beasjours S, Mori M, Hussain SNA. Regulation of cerebellar nitric oxide production in response to prolonged *in vivo* hypoxia. J Neurosci Res **49:**89–97, 1997.
28. Ichinose H, Ohye T, Matsuda Y, Hori T, Blau N, Burlina A, Rouse B, Matalon R, Fujita K, Nagatsu T. Characterization of mouse and human GTP cyclohydrolase I genes: Mutations in patients with GTP cyclohydrolase I deficiency. J Biol Chem **270:**10062–10071, 1995.
29. Witter K, Werner T, Blusch JH, Schneider EM, Riess O, Ziegler I, Rodl W, Bacher A, Gütlich M. Cloning, sequencing and functional studies of the gene encoding human GTP cyclohydrolase I. Gene **171:**285–290, 1996.
30. Schott K, Gutlich M, Ziegler I. Induction of GTP-cyclohydrolase I mRNA expression by lectin activation and γ-interferon treatment in human cells associated with the immune response. J Cell Physiol **156:**12–16, 1993.
31. Hattori Y, Gross SS. GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle: Characterization, sequence and relationship to nitric oxide synthase. Biochem Biophys Res Commun **195:**435–441, 1993.
32. Katusic ZS, Stelter A, Milstien S. Cytokines stimulate GTP cyclohydrolase I gene expression in cultured human umbilical vein endothelial cells. Arterioscler Thromb Vasc Biol **18:**27–32, 1998.
33. Hattori Y, Nakanishi N, Kasai K, Shimoda SI, Gross SS. Pyrrolidine dithiocarbamate inhibits immunostimulant-induced tetrahydrobiopterin synthesis in rat vascular smooth muscle. Eur J Pharmacol **296:**107–112, 1996.
34. Nomura T, Ohtsuki M, Matsui S, Sumiichinose C, Nomura H, Hagino Y, Iwase K, Ichinose H, Fujita K, Nagatsu T. Isolation of a full-length cDNA clone for human GTP cyclohydrolase I type 1 from pheochromocytoma. J Neural Transm Gen Sect **101:**237–242, 1995.
35. Viveros OH, Lee CL, Abou-Donia MM, Nixon JC, Nichol CA. Biopterin cofactor biosynthesis: Independent regulation of GTP cyclohydrolase in adrenal medulla and cortex. Science **213:**349–350, 1981.
36. Serova L, Nanakova B, Rivkin M, Kvetnansky R, Sabban EL. Glucocorticoids elevate GTP cyclohydrolase I mRNA levels *in vivo* and in PC12 cells. Brain Res Mol Brain Res **48:**251–258, 1997.
37. Wachter H, Fuchs D, Hausen A, Reibnegger G, Werner ER. Neopterin as marker for activation of cellular immunity: Immunologic basis and clinical application. Adv Clin Chem **27:**81–141, 1989.
38. Weiss G, Schwaighofer H, Herold M, Nachbaur D, Wachter H, Niederwieser D, Werner ER. Nitric oxide formation as predictive parameter for acute graft-versus-host disease after human allogeneic bone marrow transplantation. Transplantation **60:**1239–1244, 1995.
39. Zangerle R, Fuchs D, Reibnegger G, Werner Felmayer G, Gallati H, Wachter H, Werner ER. Serum nitrite plus nitrate in infection with human immunodeficiency virus type-1. Immunobiology **193:**59–70, 1995.
40. Groeneveld PH, Kroon FP, Nibbering PH, Bruisten SM, van Swieten P, van Furth R. Increased production of nitric oxide correlates with viral load and activation of mononuclear phagocytes in HIV-infected patients. Scand J Infect Dis **28:**341–345, 1996.
41. Strohmaier W, Werner ER, Wachter H, Redl H, Schlag G. Pteridine and nitrite/nitrate formation in experimental septic and traumatic shock. Shock **6:**254–258, 1996.
42. Werner Felmayer G, Prast H, Werner ER, Philippu A, Wachter H. Induction of GTP cyclohydrolase I by bacterial lipopolysaccharide in the rat. FEBS Lett **322:**223–226, 1993.
43. Bune AJ, Brand MP, Heales SJ, Shergill JK, Cammack R, Cook HT. Inhibition of tetrahydrobiopterin synthesis reduces *in vivo* nitric oxide production in experimental endotoxic shock. Biochem Biophys Res Commun **220:**13–19, 1996.
44. Hattori Y, Hattori S, Motohashi S, Kasai K, Shimoda SI, Nakanishi N. Co-induction of nitric oxide and tetrahydrobiopterin synthesis in the myocardium *in vivo*. Mol Cell Biochem **166:**177–181, 1997.
45. Hussain SNA, Giaid A, Eldawiri Q, Sakkal D, Hattori R, Guo Y. Expression of nitric oxide synthases and GTP cyclohydrolase I in the ventilatory and limb muscles during endotoxemia. Am J Respir Cell Mol Biol **17:**173–180, 1997.
46. van Amsterdam JG, van den Berg C, Zuidema J, te Biesebeek JD, Rokos H. Effect of septicaemia on the plasma levels of biopterin and nitric oxide metabolites in rats and rabbits. Biochem Pharmacol **52:**1447–1451, 1996.
47. Kukor Z, Meszaros G, Hertelendy F, Toth M. Calcium-dependent nitric oxide synthesis is potently stimulated by tetrahydrobiopterin in human primordial placenta. Placenta **17:**69–73, 1996.
48. Werner Felmayer G, Golderer G, Werner ER, Grobner P, Wachter H. Pteridine biosynthesis and nitric oxide synthase in *Physarum polycephalum.* Biochem J **304:**105–111, 1994.

49. Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate: Tetrahydrobiopterin is required as a cofactor. J Biol Chem **264:**19654–19658, 1989.
50. Kwon NS, Nathan CF, Stuehr DJ. Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. J Biol Chem **264:**20496–20501, 1989.
51. Mayer B, John M, Böhme E. Purification of a Ca^{2+} calmodulin-dependent nitric oxide synthase from porcine cerebellum: Cofactor-role of tetrahydrobiopterin. FEBS Lett **277:**215–219, 1990.
52. Kaufman S. New tetrahydrobiopterin-dependent systems. Annu Rev Nutr **13:**261–286, 1993.
53. Bailey SW, Dillard SB, Ayling JE. Role of C6 chirality of tetrahydropterin cofactor in catalysis and regulation of tyrosine and phenylalanine hydroxylases. Biochemistry **30:**10226–10235, 1991.
54. Klatt P, Schmid M, Leopold E, Schmidt K, Werner ER, Mayer B. The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. J Biol Chem **269:**13861–13866, 1994.
55. Werner ER, Pitters E, Schmidt K, Wachter H, Werner Felmayer G, Mayer B. Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase. Biochem J **320:**193–196, 1996.
56. Giovanelli J, Campos KL, Kaufman S. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. Proc Natl Acad Sci U S A **88:**7091–7095, 1991.
57. Xie QW, Leung M, Fuortes M, Sassa S, Nathan C. Complementation analysis of mutants of nitric oxide synthase reveals that the active site requires two hemes. Proc Natl Acad Sci U S A **93:**4891–4896, 1996.
58. Mayer B, Werner ER. In search of a function for tetrahydrobiopterin in the biosynthesis of nitric oxide. Naunyn Schmiedebergs Arch Pharmacol **351:**453–463, 1995.
59. Baek KJ, Thiel BA, Lucas S, Stuehr DJ. Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. J Biol Chem **268:**21120–21129, 1993.
60. Klatt P, Schmidt K, Lehner D, Glatter O, Bachinger HP, Mayer B. Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. EMBO J **14:**3687–3695, 1995.
61. Klatt P, Pfeiffer S, List BM, Lehner D, Glatter O, Bachinger HP, Werner ER, Schmidt K, Mayer B. Characterization of heme-deficient neuronal nitric oxide synthase reveals a role for heme in subunit dimerization and binding of the amino acid substrate and tetrahydrobiopterin. J Biol Chem **271:**7336–7342, 1996.
62. Albakri QA, Stuehr DJ. Intracellular assembly of inducible NO synthase is limited by nitric oxide–mediated changes in heme insertion and availability. J Biol Chem **271:**5414–5421, 1996.
63. List BM, Klatt P, Werner ER, Schmidt K, Mayer B. Overexpression of neuronal nitric oxide synthase in insect cells reveals requirement of haem for tetrahydrobiopterin binding. Biochem J **315:**57–63, 1996.
64. Wang J, Stuehr DJ, Rousseau DL. Tetrahydrobiopterin-deficient nitric oxide synthase has a modified heme environment and forms a cytochrome P-420 analogue. Biochemistry **34:**7080–7087, 1995.
65. McMillan K, Masters BSS. Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: Identification of the heme-binding proximal thiolate ligand as cysteine-415. Biochemistry **34:**3686–3693, 1995.
66. Rodriguez Crespo I, Gerber NC, Ortiz de Montellano PR. Endothelial nitric oxide synthase: Expression in *Escherichia coli,* spectroscopic characterization, and role of tetrahydrobiopterin in dimer formation. J Biol Chem **271:**11462–11467, 1996.
67. Crane BR, Arvai AS, Ghosh DK, Wu CQ, Getzoff ED, Stuehr DJ, Tainer JA. Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. Science **279:**2121–2126, 1998.
68. Crane BR, Arvai AS, Gachhui R, Wu CQ, Ghosh DK, Getzoff ED, Stuehr DJ, Tainer JA. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. Science **278:**425–431, 1997.
69. Renodon A, Boucher JL, Wu CQ, Gachhui R, Sari MA, Mansuy D, Stuehr D. Formation of nitric oxide synthase-iron(II) nitrosoalkane complexes: Severe restriction of access to the iron(II) site in the presence of tetrahydrobiopterin. Biochemistry **37:**6367–6374, 1998.
70. Tzeng E, Billiar TR, Robbins PD, Loftus M, Stuehr DJ. Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line:H4B promotes assembly of enzyme subunits into an active dimer. Proc Natl Acad Sci U S A **92:**11771–11775, 1995.
71. Tzeng E, Yoneyama T, Hatakeyama K, Shears LL, Billiar TR. Vascular inducible nitric oxide synthase gene therapy: Requirement for guanosine triphosphate cyclohydrolase I. Surgery **120:**315–321, 1996.
72. Riveros Moreno V, Heffernan B, Torres B, Chubb A, Charles I, Moncada S. Purification to homogeneity and characterisation of rat brain recombinant nitric oxide synthase. Eur J Biochem **230:**52–57, 1995.
73. Roman LJ, Sheta EA, Martasek P, Gross SS, Liu Q, Masters BS. High-level expression of functional rat neuronal nitric oxide synthase in *Escherichia coli.* Proc Natl Acad Sci U S A **92:**8428–8432, 1995.
74. Kullo IJ, Schwartz RS, Pompili VJ, Tsutsui M, Milstien S, Fitzpatrick LA, Katusic ZS, O'Brien T. Expression and function of recombinant endothelial NO synthase in coronary artery smooth muscle cells. Arterioscler Thromb Vasc Biol **17:**2405–2412, 1997.
75. Brand MP, Heales SJ, Land JM, Clark JB. Tetrahydrobiopterin deficiency and brain nitric oxide synthase in the hph1 mouse. J Inherit Metab Dis **18:**33–39, 1995.
76. Witteveen CF, Giovanelli J, Kaufman S. Reduction of quinonoid dihydrobiopterin to tetrahydrobiopterin by nitric oxide synthase. J Biol Chem **271:**4143–4147, 1996.
77. Stuehr DJ, Kwon NS, Nathan CF, Griffith OW, Feldman PL, Wiseman J. N ω hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J Biol Chem **266:**6259–6263, 1991.
78. Campos KL, Giovanelli J, Kaufman S. Characteristics of the nitric oxide synthase–catalyzed conversion of arginine to N-hydroxyarginine, the first oxygenation step in the enzymic synthesis of nitric oxide. J Biol Chem **270:**1721–1728, 1995.
79. Pfeiffer S, Gorren ACF, Pitters E, Schmidt K, Werner ER, Mayer B. Allosteric modulation of rat brain nitric oxide synthase by the pterin-site enzyme inhibitor 4-amino tetrahydrobiopterin. Biochem J **328:**349–352, 1997.
80. Presta A, Siddhanta U, Wu CQ, Sennequier N, Huang LX, Abusoud HM, Erzurum S, Stuehr DJ. Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis, and subunit dimerization in inducible nitric oxide synthase. Biochemistry **37:**298–310, 1998.
81. Abusoud HM, Gachhui R, Raushel FM, Stuehr DJ. The ferrous-dioxy complex of neuronal nitric oxide synthase: Divergent effects of L-arginine and tetrahydrobiopterin on its stability. J Biol Chem **272:**17349–17353, 1997.
82. Bec N, Gorren ACF, Voelker C, Mayer B, Lange R. Reaction of neuronal nitric oxide synthase with oxygen at low temperature. Evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin. J Biol Chem **273:**13502–13508, 1998.
83. Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ. Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group: Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. J Biol Chem **269:**21644–21649, 1994.
84. Mayer B, Klatt P, Werner ER, Schmidt K. Kinetics and mechanism of tetrahydrobiopterin-induced oxidation of nitric oxide. J Biol Chem **270:**655–659, 1995.
85. Abusoud HM, Wu CQ, Ghosh DK, Stuehr DJ. Stopped-flow analysis of CO and NO binding to inducible nitric oxide synthase. Biochemistry **37:**3777–3786, 1998.
86. Cho HJ, Martin E, Xie QW, Sassa S, Nathan C. Inducible nitric oxide synthase: Identification of amino acid residues essential for dimerization and binding of tetrahydrobiopterin. Proc Natl Acad Sci U S A **92:**11514–11518, 1995.
87. Boyhan A, Smith D, Charles IG, Saqi M, Lowe PN. Delineation of the arginine- and tetrahydrobiopterin-binding sites of neuronal nitric oxide synthase. Biochem J **323:**131–139, 1997.
88. Gosh DK, Wu C, Pitters E, Moloney M, Werner ER, Mayer B, Stuehr DJ. Characterization of the inducible nitric oxide synthase oxygenase domain identifies a 49-amino acid segment required for subunit dimerization and tetrahydrobiopterin binding. Biochemistry **36:**10609–10619, 1997.
89. Venema RC, Ju H, Zou R, Ryan JW, Venema VJ. Subunit interactions of endothelial nitric oxide synthase: Comparisons to the neuro-

nal and inducible nitric oxide synthase isoforms. J Biol Chem **272:**1276–1282, 1997.
90. van Amsterdam JG, Wemer J. Tetrahydrobiopterin induces vasodilation *via* enhancement of cGMP level. Eur J Pharmacol **215:**349–350, 1992.
91. Tsutsui M, Milstien S, Katusic ZS. Effect of tetrahydrobiopterin on endothelial function in canine middle cerebral arteries. Circ Res **79:**336–342, 1996.
92. Rosenblum WI. Tetrahydrobiopterin, a cofactor for nitric oxide synthase, produces endothelium-dependent dilation of mouse pial arterioles. Stroke **28:**186–189, 1997.
93. Tiefenbacher CP, Chilian WM, Mitchell M, DeFily DV. Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. Circulation **94:**1423–1429, 1996.
94. Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. Circulation **91:**139–144, 1995.
95. Heinzel B, John M, Klatt P, Bohme E, Mayer B. Ca^{2+}/calmodulin–dependent formation of hydrogen peroxide by brain nitric oxide synthase. Biochem J **281:**627–630, 1992.
96. List BM, Klosch B, Volker C, Gorren AC, Sessa WC, Werner ER, Kukovetz WR, Schmidt K, Mayer B. Characterization of bovine endothelial nitric oxide synthase as a homodimer with downregulated uncoupled NADPH oxidase activity: Tetrahydrobiopterin binding kinetics and role of haem in dimerization. Biochem J **323:**159–165, 1997.
97. Wever RMF, Vandam T, Vanrijn HJM, Degroot F, Rabelink TJ. Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. Biochem Biophys Res Commun **237:**340–344, 1997.
98. Liochev SI, Fridovich I. Lucigenin (bis-N-methylacridinium) as a mediator of superoxide anion production. Arch Biochem Biophys **337:**115–120, 1997.
99. Vasquezvivar J, Hogg N, Pritchard KA, Martasek P, Kalyanaraman B. Superoxide anion formation from lucigenin: An electron spin resonance spin-trapping study. FEBS Lett **403:**127–130, 1997.
100. Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest **99:**41–46, 1997.
101. Cosentino F, Sill JC, Katusic ZS. Role of superoxide anions in the mediation of endothelium-dependent contractions. Hypertension **23:**229–235, 1994.
102. Valen G, Skjelbakken T, Vaage J. The role of nitric oxide in the cardiac effects of hydrogen peroxide. Mol Cell Biochem **159:**7–14, 1996.
103. Shiraki T, Koshimura K, Kobayashi S, Miwa S, Masaki T, Watanabe Y, Murakami Y, Kato Y. Stimulating effect of 6R-tetrahydrobiopterin on Ca^{2+} channels in neurons of rat dorsal motor nucleus of the vagus. Biochem Biophys Res Commun **221:**181–185, 1996.
104. Cosentino F, Patton S, Duscio LV, Werner ER, Wernerfelmayer G, Moreau P, Malinski T, Luscher TF. Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. J Clin Invest **101:**1530–1537, 1998.
105. Verhaar MC, Wever RMF, Kastelein JJP, Vandam T, Koomans HA, Rabelink TJ. 5-Methyltetrahydrofolate, the active form of folic acid, restores endothelial function in familial hypercholesterolemia. Circulation **97:**237–241, 1998.
106. Hornig B, Arakawa N, Kohler C, Drexler H. Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. Circulation **97:**363–368, 1998.
107. Pritchard KAJ, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. Circ Res **77:**510–518, 1995.
108. Werner ER, Werner Felmayer G, Fuchs D, Hausen A, Reibnegger G, Wachter H. Parallel induction of tetrahydrobiopterin biosynthesis and indoleamine 2,3-dioxygenase activity in human cells and cell lines by γ-interferon. Biochem J **262:**861–866, 1989.
109. Hofmann B, Bass H, Nishanian P, Faisal M, Figlin RA, Sarna GP, Fahey JL. Different lymphoid cell populations produce varied levels of neopterin, beta 2-microglobulin and soluble IL-2 receptor when stimulated with IL-2, γ-interferon or tumour necrosis factor-α. Clin Exp Immunol **88:**548–554, 1992.
110. Werner Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Schmidt K, Weiss G, Wachter H. Pteridine biosynthesis in human endothelial cells: Impact on nitric oxide–mediated formation of cyclic GMP. J Biol Chem **268:**1842–1846, 1993.
111. Rosenkranz Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. Elevations in tetrahydrobiopterin levels enhance endothelial nitric oxide synthase specific activity. J Clin Invest **93:**2236–2243, 1994.
112. Schoedon G, Schneemann M, Blau N, Edgell CJ, Schaffner A. Modulation of human endothelial cell tetrahydrobiopterin synthesis by activating and deactivating cytokines. New perspectives on endothelium-derived relaxing factor. Biochem Biophys Res Commun **196:**1343–1348, 1993.
113. Werner ER, Werner Felmayer G, Fuchs D, Hausen A, Reibnegger G, Yim JJ, Pfleiderer W, Wachter H. Tetrahydrobiopterin biosynthetic activities in human macrophages, fibroblasts, THP-1, and T 24 cells: GTP-cyclohydrolase I is stimulated by γ-interferon, and 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase are constitutively present. J Biol Chem **265:**3189–3192, 1990.
114. Ziegler I, Schott K, Lubbert M, Herrmann F, Schwulera U, Bacher A. Control of tetrahydrobiopterin synthesis in T lymphocytes by synergistic action of γ-interferon and interleukin-2. J Biol Chem **265:**17026–17030, 1990.
115. Gütlich M, Schott K, Werner T, Bacher A, Ziegler I. Species and tissue specificity of mammalian GTP cyclohydrolase-I messenger RNA. Biochim Biophys Acta **1171:**133–140, 1992.
116. Huber C, Batchelor JR, Fuchs D, Hausen A, Lang A, Niederwieser D, Reibnegger G, Swetly P, Troppmair J, Wachter H. Immune response–associated production of neopterin: Release from macrophages primarily under control of γ-interferon. J Exp Med **160:**310–316, 1984.
117. Schoedon G, Troppmair J, Adolf G, Huber C, Niederwieser A. γ-Interferon enhances biosynthesis of pterins in peripheral blood mononuclear cells by induction of GTP-cyclohydrolase I activity. J Interferon Res **6:**697–703, 1986.
118. Troppmair J, Nachbaur K, Herold M, Aulitzky W, Tilg H, Gastl G, Bieling P, Kotlan B, Flener R, Mull B, Rokos H, Huber C. *In vitro* and *in vivo* studies on the induction of neopterin biosynthesis by cytokines, alloantigens and lipopolysaccharide (LPS). Clin Exp Immunol **74:**392–397, 1988.
119. Werner Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter H. Tumour necrosis factor-α and lipopolysaccharide enhance interferon-induced tryptophan degradation and pteridine synthesis in human cells. Biol Chem Hoppe Seyler **370:**1063–1069, 1989.
120. Huber C, Fuchs D, Hausen A, Margreiter R, Reibnegger G, Spielberger M, Wachter H. Pteridines as a new marker to detect human T cells activated by allogeneic or modified self major histocompatibility complex (MHC) determinants. J Immunol **130:**1047–1050, 1983.
121. Ziegler I. Synthesis and γ-interferon-controlled release of pteridines during activation of human peripheral blood mononuclear cells. Biochem Biophys Res Commun **132:**404–411, 1985.
122. Ziegler I, Schwulera U, Ellwart J. Pteridines are produced during interleukin 2-induced T-cell proliferation and modulate transmission of this signal. Exp Cell Res **167:**531–538, 1986.
123. Blau N, Joller P, Atares M, Cardesa-Garcia J, Niederwieser A. Increase of GTP cyclohydrolase I activity in mononuclear blood cells by stimulation: Detection of heterozygotes of GTP cyclohydrolase I deficiency. Clin Chim Acta **148:**47–52, 1985.
124. Werner Felmayer G, Werner ER, Fuchs D, Hausen A, Mayer B, Reibnegger G, Weiss G, Wachter H. Ca^{2+}/calmodulin-dependent nitric oxide synthase activity in the human cervix carcinoma cell line ME-180. Biochem J **289:**357–361, 1993.
125. Walter R, Linscheid P, Blau N, Kierat L, Schaffner A, Schoedon G. Induction of tetrahydrobiopterin synthesis in human umbilical vein smooth muscle cells by inflammatory stimuli. Immunol Lett **60:**13–17, 1998.
126. Kasai K, Hattori Y, Nakanishi N, Manaka K, Banba N, Motohashi S, Shimoda S. Regulation of inducible nitric oxide production by cytokines in human thyrocytes in culture. Endocrinology **136:**4261–4270, 1995.
127. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter H. Neopterin formation and tryptophan degradation by a

human myelomonocytic cell line (THP-1). Cancer Res **50:**2863–2867, 1990.
128. Sakai N, Kaufman S, Milstien S. Parallel induction of nitric oxide and tetrahydrobiopterin synthesis by cytokines in rat glial cells. J Neurochem **65:**895–902, 1995.
129. Werner ER, Werner Felmayer G, Fuchs D, Hausen A, Reibnegger G, Yim JJ, Wachter H. Impact of tumour necrosis factor-α and γ-interferon on tetrahydrobiopterin synthesis in murine fibroblasts and macrophages. Biochem J **280:**709–714, 1991.
130. Hattori Y, Nakanishi N. Effects of cyclosporin A and FK506 on nitric oxide and tetrahydrobiopterin synthesis in bacterial lipopolysaccharide-treated J774 macrophages. Cell Immunol **165:**7–11, 1995.
131. Schoedon G, Troppmair J, Fontana A, Huber C, Curtius HC, Niederwieser A. Biosynthesis and metabolism of pterins in peripheral blood mononuclear cells and leukemia lines of man and mouse. Eur J Biochem **166:**303–310, 1987.
132. Yoshida S, Ota A, Umezawa K, Nagatsu T. Dissociated release of tetrahydrobiopterin and nitric oxide by lipopolysaccharide from mouse neuroblastoma cells. Neurosci Lett **212:**135–138, 1996.
133. Sakai N, Kaufman S, Milstein S. Tetrahydrobiopterin is required for cytokine-induced nitric oxide production in a murine macrophage cell line (RAW 264). Mol Pharmacol **43:**6–10, 1993.
134. D'Sa C, Hirayama K, West A, Hahn M, Zhu M, Kapatos G. Tetrahydrobiopterin biosynthesis in C6 glioma cells. Induction of GTP cyclohydrolase I gene expression by lipopolysaccharide and cytokine treatment. Brain Res Mol Brain Res **41:**105–110, 1996.
135. Tabraue C, Diaz Penate R, Gallardo G, Hernandez I, Quintana J, Lopez Blanco F, Gonzalez Reyes J, Fanjul LF, Ruiz de Galarreta CM. Induction of guanosine triphosphate-cyclohydrolase by follicle-stimulating hormone enhances interleukin-1 β-stimulated nitric oxide synthase activity in granulosa cells. Endocrinology **138:**162–168, 1997.
136. Di Silvio M, Geller DA, Gross SS, Nussler A, Freeswick P, Simmons RL, Billiar TR. Inducible nitric oxide synthase activity in hepatocytes is dependent on the coinduction of tetrahydrobiopterin synthesis. Adv Exp Med Biol **338:**305–308, 1993.
137. Mühl H, Pfeilschifter J. Tetrahydrobiopterin is a limiting factor of nitric oxide generation in interleukin-1 β-stimulated rat glomerular mesangial cells. Kidney Int **46:**1302–1306, 1994.
138. Plüss C, Werner ER, Blau N, Wachter H, Pfeilschifter J. Interleukin-1β and cAMP trigger the expression of GTP cyclohydrolase I in rat renal mesangial cells. Biochem J **318:**665–671, 1996.
139. Hirahashi J, Nakaki T, Hishikawa K, Marumo T, Yasumori T, Hayashi M, Suzuki H, Saruta T. Endothelin-1 inhibits induction of nitric oxide synthase and GTP cyclohydrolase I in rat mesangial cells. Pharmacology **53:**241–249, 1996.
140. Anastasiadis PZ, Kuhn DM, Blitz J, Imerman BA, Louie MC, Levine RA. Regulation of tyrosine hydroxylase and tetrahydrobiopterin biosynthetic enzymes in PC12 cells by NGF, EGF, and IFN-γ. Brain Res **713:**125–133, 1996.
141. Scott Burden T, Elizondo E, Ge T, Boulanger CM, Vanhoutte PM. Growth factor regulation of interleukin-1 β–induced nitric oxide synthase and GTP: Cyclohydrolase expression in cultured smooth muscle cells. Biochem Biophys Res Commun **196:**1261–1266, 1993.
142. Walter R, Blau N, Kierat L, Schaffner A, Schoedon G. Effects of activating and deactivating cytokines on the functionally linked tetrahydrobiopterin: No pathways in vascular smooth muscle cells. Immunol Lett **54:**25–29, 1996.
143. Hattori Y, Kasai K, Nakanishi N, Gross SS, Thiemermann C. Induction of nitric oxide and tetrahydrobiopterin synthesis by lipoteichoic acid from *Staphylococcus aureus* in vascular smooth muscle cells. J Vasc Res **35:**104–108, 1998.
144. Stegenga SL, Hirayama K, Kapatos G. Regulation of GTP cyclohydrolase I gene expression and tetrahydrobiopterin content in cultured sympathetic neurons by leukemia inhibitory factor and ciliary neurotrophic factor. J Neurochem **66:**2541–2545, 1996.
145. Sung YJ, Hotchkiss JH, Dietert RR. 2,4-Diamino-6-hydroxypyrimidine, an inhibitor of GTP cyclohydrolase I, suppresses nitric oxide production by chicken macrophages. Int J Immunopharmacol **16:**101–108, 1994.
146. Kaspers B, Gutlich M, Witter K, Losch U, Goldberg M, Ziegler I. Coordinate induction of tetrahydrobiopterin synthesis and nitric oxide synthase activity in chicken macrophages: Upregulation of GTP cyclohydrolase I activity. Comp Biochem Physiol [B] **117:**209–215, 1997.
147. Gross SS, Jaffe EA, Levi R, Kilbourn RG. Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent, and inhibited by arginine analogs with a rank order of potency characteristic of activated macrophages. Biochem Biophys Res Commun **178:**823–829, 1991.
148. Amoah Apraku B, Tang SS, Ingelfinger JR, Guzman NJ. Guanosine triphosphate cyclohydrolase I regulates nitric oxide synthesis in renal proximal tubules. J Am Soc Nephrol **5:**1630–1633, 1995.
149. Schmidt K, Werner ER, Mayer B, Wachter H, Kukovetz WR. Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. Biochem J **281:**297–300, 1992.
150. Demerle Pallardy C, Lonchampt MO, Chabrier PE, Braquet P. Nitric oxide synthase induction in glial cells: Effect on neuronal survival. Life Sci **52:**1883–1890, 1993.
151. Jorens PG, Van Overveld FJ, Vermeire PA, Bult H, Herman AG. Synergism between interleukin-1β and γ-interferon, an inducer of nitric oxide synthase, in rat lung fibroblasts. Eur J Pharmacol **224:**7–12, 1992.
152. Nakayama DK, Geller DA, Di Silvio M, Bloomgarden G, Davies P, Pitt BR, Hatakeyama K, Kagamiyama H, Simmons RL, Billiar TR. Tetrahydrobiopterin synthesis and inducible nitric oxide production in pulmonary artery smooth muscle. Am J Physiol **266:**L455–L460, 1994.
153. Cubberley RR, Alderton WK, Boyhan A, Charles IG, Lowe PN, Old RW. Cysteine-200 of human inducible nitric oxide synthase is essential for dimerization of haem domains and for binding of haem, nitroarginine, and tetrahydrobiopterin. Biochem J **323:**141–146, 1997.
154. Chen PF, Tsai AL, Wu KK. Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. Biochem Biophys Res Commun **215:**1119–1129, 1995.