MINIREVIEW

Tetrahydrobiopterin and Cytokines (43566A)

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Abstract. **Biosynthesis of tetrahydrobiopterin starts from guanosine triphosphate by the action of guanosine triphosphate cyclohydrolase I, which yields the first intermediate, 7,8-dihydroneopterin triphosphate. This compound is then converted by subsequent enzymes, 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase, to tetrahydrobiopterin, the biologically active metabolite. Cytokines such as γ-interferon or tumor necrosis factor-α strongly stimulate the activity of guanosine triphosphate cyclohydrolase I in murine and human cells, yielding a potentiation of intracellular tetrahydrobiopterin concentrations. In human cells, particularly in human monocytes and macrophages, the low activity of 6-pyruvoyl tetrahydropterin synthase leads to the additional accumulation of neopterin derivatives, which leak from the cells after dephosphorylation and are found increased in body fluids of humans with diseases challenging cell-mediated immunity. A functional role for the stimulation of tetrahydrobiopterin biosynthesis by cytokines is the formation of a limiting cofactor required for the enzymatic conversion of L-arginine to citrulline and nitric oxide.** [P.S.E.B.M. 1993, Vol 203]

Pteridines are a class of compounds with a common heterocyclic ring structure, the pyrazino[2,3-*d*] pyrimidine (Fig. 1). 2-Amino-4-hydroxypteridines are called pterins, whereas the 2,4-dihydroxy derivatives are called lumazines. Pteridines include vitamins, such as folic acid and riboflavin, as well as several compounds endogenously synthesized in mammals, such as neopterin and biopterin derivatives. Pteridines were first characterized as pigments in the wings of butterflies (1, 2). The knowledge on some of the compounds is still limited, e.g., on the biosynthesis of molybdopterin, a cofactor of xanthine oxidase and sulphite oxidase (3, 4). Biopterin was isolated from 4000 liters of human urine, and an observed biologic effect was the support of the growth of *Chrithidia fasciculata* (5), a protozoon. Subsequently, defined biochemical roles of the 5,6,7,8-tetrahydroderivative of biopterin were found as cofactor in the monooxygenation of phenylalanine (6), tyrosine (7), and tryptophan (8). In addition, the oxidative cleavage of glyceryl ethers was observed to require a tetrahydropterin (9, 10). The biosynthesis of tetrahydrobiopterin was discovered to start from guanosine triphosphate (GTP) by action of GTP cyclohydrolase I (11, reviewed in 12), yielding the first intermediate, 7,8-dihydroneopterin triphosphate. Several groups then concluded that the further steps from 7,8-dihydroneopterin triphosphate to tetrahydrobiopterin, the endproduct of the pathway and the biologically active metabolite, proceed via tetrahydroderivatives (reviewed in 13). At least two further enzymes are involved, i.e., 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase (see below for details).

Inherited defects of the enzymes synthesizing tetrahydrobiopterin from GTP or recycling the cofactor after the hydroxylation to its active tetrahydroform were discovered (14, reviewed in 15). These are known as "atypical phenylketonuria," since in these disorders the catabolism of phenylalanine is impaired due to the lack of the active cofactor, and not due to a mutation of the phenylalanine monooxygenase itself as in the "typical" form. In accordance with the biochemical findings, not only the catabolism of phenylalanine, but

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also the formation of serotonin and dopamine was found to be impaired in these rare inherited disorders (15).

Several puzzling observations then indicated that this is not yet the whole picture. Tetrahydrobiopterin biosynthesis was detected in regions lacking activities of the known pteridine-dependent hydroxylases (16). Cultured fibroblasts showed some of the enzyme activities required for biosynthesis and recycling of tetrahydrobiopterin, but apparently did not synthesize this metabolite (17). Patients suffering from viral infections and from tumors showed increased excretion of neopterin (18, reviewed in 19–21), which had also been observed in a metabolic defect of the enzymes synthesizing tetrahydrobiopterin from 7,8-dihydroneopterin triphosphate (22). In contrast to this metabolic defect, however, biopterin derivatives were found somewhat increased rather than decreased in infectious diseases (23).

Previous papers have compiled the knowledge on the biosynthesis of tetrahydrobiopterin and molybdopterin (13), on the metabolic role of tetrahydrobiopterin (24), on GTP cyclohydrolases (12), on inborn errors of tetrahydrobiopterin metabolism (15), on the physiologic and clinical chemistry of biopterin (25), on the strategy for screening of tetrahydrobiopterin deficiency (26), as well as on the excretion patterns of neopterin in various clinical conditions (19–21). The present Minireview will summarize shortly more recent advances in the field, with special emphasis on the stimulation of tetrahydrobiopterin biosynthesis by cytokines and a reaction which the increased intracellular tetrahydrobiopterin is required for, i.e., the enzymatic conversion of L-arginine to citrulline and nitric oxide.

Tetrahydrobiopterin Biosynthesis

Figure 2 shows a simplified scheme of tetrahydrobiopterin synthesis. As already mentioned above, the biosynthesis starts from guanosine 5′-triphosphate, which is cleaved to the first intermediate 7,8-dihydroneopterin 3′-triphosphate by GTP cyclohydrolase I (EC 3.5.4.16). This is then converted by 6-pyruvoyl tetrahydropterin synthase to 6-pyruvoyl tetrahydropterin, the structure of which has recently been confirmed by spectroscopic techniques (27). Recent work has also

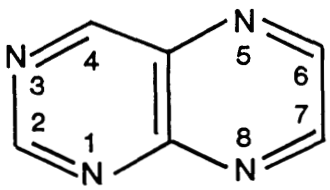

Figure 1. Structure of the pteridine nucleus. 2-Amino-4-hydroxy derivatives are called pterins. 2,4-Dihydroxy derivatives are called lumazines. Many naturally occurring pterins have a side chain in Position 6.

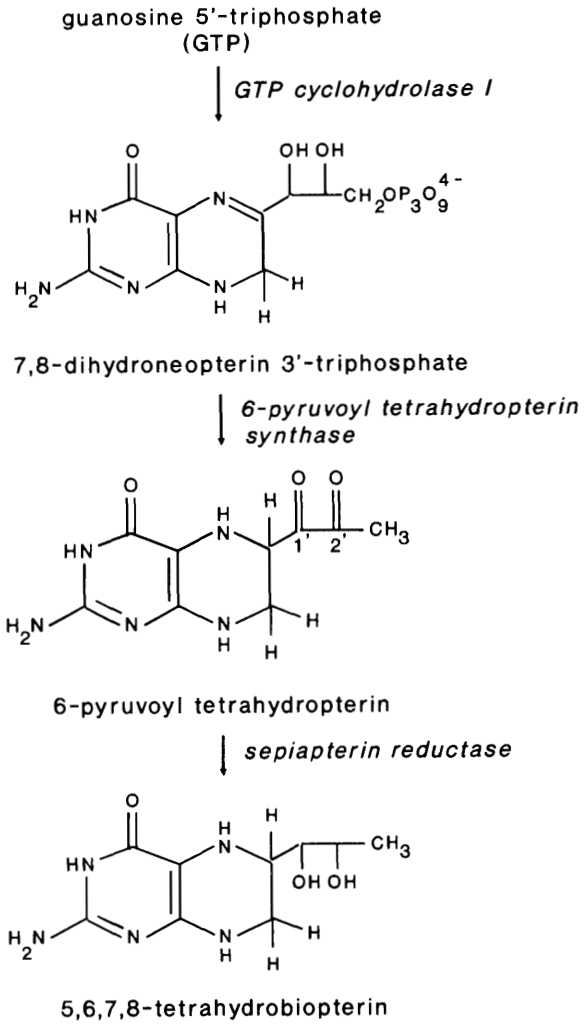

Figure 2. Simplified scheme of biosynthesis of tetrahydrobiopterin from GTP. The reduction of the 1′- and 2′-oxo groups of 6-pyruvoyl tetrahydropterin can be carried out by sepiapterin reductase at the expense of NADPH. It is not clear at present whether additional reductases are involved in this step (see text for detailed discussion). Whereas GTP cyclohydrolase I does not require any cofactor (12), Mg(2+) is essential for the 6-pyruvoyl tetrahydropterin synthase reaction (33, 42).

focused on the precise mechanism of the reduction of the 1′- and 2′-oxo groups to the respective hydroxyl groups. An enzyme reducing the 2′-oxo group has been purified from rat brain (28) and from human liver, which was shown to be identical to aldose reductase (29). Using neutralizing antibodies, some participation of this so-called 6-pyruvoyl tetrahydropterin reductase enzyme in tetrahydrobiopterin biosynthesis was seen in rat brain but not in rat liver (30). Sepiapterin reductase (EC 1.1.1.153) can perform the reductions of both 1′- and 2′-oxo functions, so that an additional reductase may only be needed when sepiapterin reductase becomes limiting (28) or is depleted by a genetic defect (31). Inhibition of sepiapterin reductase by an antiserum led to accumulation of 6-lactoyl tetrahydropterin (1′-oxo-2′-hydroxy tetrahydropterin) in one study (32),

which was not observed in a previous investigation (33). Thus, the precise sequence of reductions of the 1′- and 2′-oxo function of 6-pyruvoyl tetrahydropterin and the requirement for an additional 6-pyruvoyl tetrahydropterin reductase remain controversial. For clarity, only sepiapterin reductase, which is generally agreed to be involved in the pathway, is shown in Figure 2. GTP cyclohydrolase I (34–36), 6-pyruvoyl tetrahydropterin synthase (37, 38), and sepiapterin reductase (39–41) have recently been sequenced and cloned. GTP cyclohydrolase I does not require any additional cofactors, whereas the activity of 6-pyruvoyl tetrahydropterin synthase depends on Mg(2+) (42, 43), and NADPH is used as coenzyme for the reduction of the keto groups of 6-pyruvoyl tetrahydropterin (43).

Stimulation of Tetrahydrobiopterin Biosynthesis by Cytokines

Research on the stimulation of pteridine synthesis by cytokines started with the finding of increased urinary excretion of neopterin in patient suffering from tumors and from viral infections (18). More detailed clinical studies of neopterin excretion patterns then showed a picture compatible with the hypothesis that neopterin is formed in connection with the activation of cell-mediated immunity (reviewed in 19–21). For example, increased excretion of neopterin was observed during allograft rejection (44). In parallel to these *in vivo* observations, *in vitro* experiments revealed that neopterin is released by activated, but not by unstimulated human peripheral blood mononuclear cells (45, 46). Cellular fractionation then showed that neopterin is formed by macrophages primarily under control of γ-interferon, which is turn is derived from activated T lymphocytes (47). In homogenates of γ-interferon-treated macrophages, increased activity of GTP cyclohydrolase I was observed (48). However, neither 7,8-dihydroneopterin triphosphate nor tetrahydrobiopterin were detected in human macrophages by the analytical methods available at this time (48). The lack of tetrahydrobiopterin detection combined with the increase of GTP following the γ-interferon treatment then led Schoedon *et al.* (49) to the conclusion that the increased accumulation of neopterin in human macrophages was due to a lack of feedback inhibition on GTP cyclohydrolase I by tetrahydrobiopterin, rather than due to increased biosynthetic activity.

Using a more sensitive analytical approach (50), however, we were then able to show that human macrophages also synthesize tetrahydrobiopterin in addition to neopterin derivatives (51). In particular, increased intracellular concentrations of both, neopterin and biopterin derivates, were found not only in macrophages treated with γ-interferon, but also in a set of human cells and cell lines of various tissue origin treated with this cytokine (51). More than 90% of the biopterin derivatives occurred in the biologically active tetrahydroform. Whereas neopterin derivatives leak from the cells after dephosphorylation (52), tetrahydrobiopterin is efficiently retained in the cells, and biopterin derivatives accumulate in the supernatant only to a much lesser extent than do neopterin derivatives (51). This can explain the above-mentioned observation, that the high increase of neopterin in infectious diseases is paralleled only by a moderate, insignificant increase in biopterin derivatives (23). γ-Interferon led to a time-dependent, up to 100-fold increase of GTP cyclohydrolase I activity in the protein fractions of cell homogenates (51, 52). The precise mechanism of the stimulation of GTP cyclohydrolase I by cytokines remains to be demonstrated. The long time (about 24 hr) consumed to reach maximal activity (52) suggests that γ-interferon induces *de novo* synthesis of GTP cyclohydrolase I protein. 6-Pyruvoyl tetrahydropterin synthase and sepiapterin reductase activities were constitutively present in all cells investigated (52) and remained unaffected by the interferon treatment. The ratio of neopterin to biopterin derivatives ranged from 52:1 in macrophages to 1:7 in dermal fibroblasts and 1:50 in T 24 cells (51). This was in line with the activities of the induced GTP cyclohydrolase I relative to the constitutive 6-pyruvoyl tetrahydropterin synthase, which was found to be particularly low in human monocytes and macrophages (52). Nevertheless, also in human macrophages, the action of γ-interferon controls the amount of tetrahydrobiopterin formed by regulating GTP cyclohydrolase I activity. The reason for this is the comparatively high K_m of 6-pyruvoyl tetrahydropterin synthase for its substrate, 7,8-dihydroneopterin triphosphate, which in human liver (42) as well as in cultured human T 24 cells (53) was determined to be 10 μM. Figure 3 shows the high-performance liquid chromatography profiles of γ-interferon-treated and untreated human macrophages illustrating the increased formation of tetrahydrobiopterin by human macrophages after the stimulation with γ-interferon. In addition, 3-hydroxyanthranilic acid (see below) and reduced forms of neopterin and 6-threo-1′2′3′-trihydroxypropylpterin are detected in homogenates of stimulated macrophages. The achiral column can not distinguish between the two enantiomers of 6-threo-1′2′3′-trihydroxypropylpterin. These are called monapterin, which occurs in microorganisms (54), and umanopterin, which has been isolated from the urine of cancer patients (55). Comparison with the human myelomonocytic cell line THP-1, which shows pteridine patterns (56) and 6-pyruvoyl tetrahydropterin synthase activities (52) similar to human macrophages isolated from peripheral blood, indicates that tetrahydrobiopterin is formed by cells of the monocytic lineage and does not originate from an impurity of the macrophage preparation.

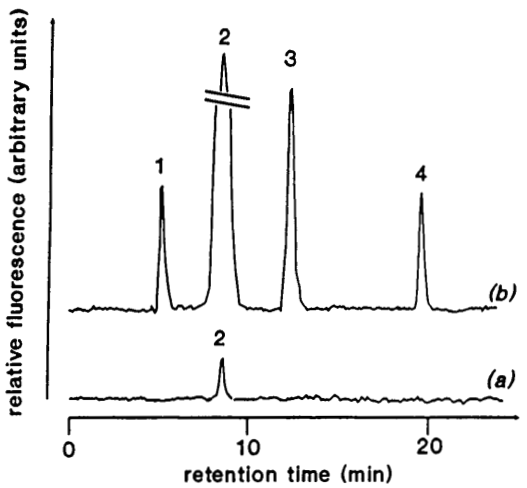

Figure 3. High-performance liquid chromatograms of pteridines in homogenates of human macrophages. (*a*) Untreated cells; (*b*) cells treated with 250 units/ml of γ-interferon for 72 hr. Cells were harvested by scraping off the plate, homogenates were prepared, and reduced forms of pteridines were oxidized with iodine in acidic solution to the fluorescent, fully aromatic forms and subjected to reversed-phase high-performance liquid chromatography with fluorescence detection as detailed in Ref. 51. Peaks: 1, 3-hydroxyanthranilic acid; 2, neopterin; 3, 6-threo-1′2′3′-trihydroxypropylpterin (the achiral column can not distinguish between the two enantiomers monapterin and umanopterin (55); and 4, biopterin.

In parallel to these studies, pteridine synthesis was investigated in murine (57) and human (58) peripheral blood mononuclear cells stimulated by lectins, leading to increased formation of biopterin, 6-hydroxymethylpterin and 6-formylpterin in the murine cells, and to accumulation of neopterin, biopterin, and 6-hydroxymethylpterin in the human cells. This system is complex, since lectins selectively induce proliferation in only some cell types of the mixture, and in addition trigger formation of cytokines, such as γ-interferon, which in turn stimulate pteridine synthesis (47). Nevertheless, some results were found similar to studies mentioned above, which in contrast were done in experimental settings minimizing proliferation (51, 52). GTP cyclohydrolase I was observed to be stimulated upon activation of the cells (59, 60); sepiapterin reductase and 6-pyruvoyl tetrahydropterin synthase remained unaffected in some of the experiments, but were stimulated in others (60, 61). On the other hand, a human natural killer such as T cell line was described (YT), which constitutively expresses GTP cyclohydrolase I activity, lacks 6-pyruvoyl tetrahydropterin synthase, and expresses sepiapterin reductase activity only when incubated with sepiapterin (62). γ-Interferon and interleukin 2 did not alter the GTP cyclohydrolase I activity in YT cells. It is not clear from these experiments, however, whether other actions of cytokines, e.g., major histocompatibility complex antigen expression, were intact in this case, or whether the cells did not react to the cytokines at all.

The kind of cytokines or agents that can stimulate GTP cyclohydrolase I activity depends upon the cell and species used. In human cells, γ-interferon is the most active stimulus (47, 56, 63–66). In the presence of T lymphocytes, many agents can act in an indirect way by triggering formation of γ-interferon and other cytokines from T lymphocytes, which then cause pteridine formation by other cells, e.g., macrophages. An example of this is the lectin phytohemagglutinin, which stimulates T cells to produce lymphokines, which then can stimulate neopterin production by macrophages. Neutralization of the stimulatory activity by antibodies to γ-interferon, but not to α-interferon, subtypes underlined the importance of γ-interferon as a stimulatory cytokine in this case (47). In human peripheral blood mononuclear cells, α- and β-interferons can increase neopterin production indirectly by triggering the formation of a 15-kDa protein, which then causes formation of γ-interferon (67). Human macrophages (63–65), human THP-1 myelomonocytoma (56), and human umbilical vein endothelial cells (68) also respond to treatment with bacterial lipopolysaccharide (LPS) by increasing GTP cyclohydrolase I activity. In the presence of T lymphocytes, however, LPS acts by triggering production of γ-interferon and other cytokines, which then cause the stimulation of GTP cyclohydrolase I activity. This was deduced from the influence of dexamethasone, which costimulates pteridine synthesis in macrophages, but in the additional presence of lymphocytes, it suppresses the LPS-induced pteridine synthesis by decreasing the amount of γ-interferon formed (65). Human recombinant α2- (47), αD- (63), α2b-, and β (65)-interferons also stimulate neopterin formation in macrophages. Since the myelomonocytic cell line THP-1 behaves similarly (56), it is clear that this is a direct effect on monocytes/macrophages that is not mediated by residual cells contaminating the macrophage cultures. Factors not causing neopterin production in human macrophages are colony-stimulating factor 1, granulocyte-monocyte colony-stimulating factor, natural α-interferon, recombinant αA-interferon (63), zymosan, phytohemagglutinin, and concanavalin A (47). Tumor necrosis factor-α stimulates GTP cyclohydrolase I in human and in murine (69) fibroblasts (Table I), whereas it is inactive as a single stimulus on human macrophages. However, tumor necrosis factor-α is a costimulus for γ-interferon in human macrophages (64, 65) and human fibroblasts (65). Costimulation of the γ-interferon action was also observed with LPS (64, 65) and dexamethasone (65). It is probable that a number of other as yet not tested cytokines might be active in costimulating to γ-interferon. Free and transferrin-bound iron attenuated the stimulatory action of a given dose of γ-interferon in THP-1 cells (70). In human peripheral blood mononuclear cells (71), as well as in a murine T cell line (49), interleukin 2 was found to

Table I. Comparison of the Influence of Tumor Necrosis Factor-α on Tetrahydrobiopterin Biosynthetic Activities and on Pteridine Levels in Human and in Murine Fibroblasts[a]

	Murine fibroblasts		Human fibroblasts	
	Control	TNF-α	Control	TNF-α
GTP cyclohydrolase I (pmol mg^{-1} min^{-1})	0.05 ± 0.01	1.52 ± 0.05	0.02 ± 0.01	0.62 ± 0.02
6-Pyruvoyl tetrahydropterin synthase (pmol mg^{-1} min^{-1})	39.2 ± 3.1	35.8 ± 1.7	0.61 ± 0.05	0.95 ± 0.09
Sepiapterin reductase (pmol mg^{-1} min^{-1})	451 ± 31	466 ± 18	567 ± 31	583 ± 17
Neopterin (pmol mg^{-1})	<1	<1	<1	15 ± 3
Biopterin (pmol mg^{-1})	10 ± 3	134 ± 18	<2	49 ± 8

[a] Normal human and murine fibroblasts were obtained from skin biopsies, cultivated as confluent monolayers, and treated with 500 units/ml of murine or human tumor necrosis factor-α (TNF-α) for 72 hr. Cells were harvested by trypsinization. Homogenates were prepared by freezing and thawing, and enzyme activities and intracellular concentrations of pteridines were determined as detailed in Ref. 52.

increase pteridine synthesis. Subsequently, γ-interferon was identified to be an essential factor for the stimulation of tetrahydrobiopterin synthesis by interleukin 2 in the transformed human T cell line MT-2 (61). In B cells, interleukin 2, γ-interferon, and pokeweed mitogen were shown to stimulate neopterin formation (66). Phorbol esters were reported to lead to a short-term, transient pteridine accumulation in human cells and cell lines (72).

A point of interest in the context of stimulation of tetrahydrobiopterin biosynthesis by cytokines is the comparison of human and murine cells. Among mammals, primates are unique in the occurrence of substantial amounts of neopterin derivatives in addition to biopterin in body fluids and tissues (16). Experiments with cultures of murine cells (69) revealed that compared with human cells γ-interferon is less potent in activating tetrahydrobiopterin synthesis. Untreated murine macrophages and macrophage lines express considerable basal GTP cyclohydrolase I activities, which are only slightly stimulated by combinations of γ-interferon and tumor necrosis factor-α. A comparison of the behavior of murine (69) and human fibroblasts to stimulation with tumor necrosis factor-α indicates that stimulation of pteridine synthesis by cytokines can occur in a similar way in human and in murine cells (Table I). GTP cyclohydrolase I is stimulated about 30-fold in both cell types, whereas 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase are already present in control cells and remain largely unaffected by the cytokine treatment. A major difference is the much lower 6-pyruvoyl tetrahydropterin synthase activity in human fibroblasts. 7,8-Dihydroneopterin triphosphate formed by GTP cyclohydrolase I is, therefore, much less efficiently converted to tetrahydrobiopterin, allowing phosphatases to cleave a portion of this intermediate to 7,8-dihydroneopterin, which is not further metabolized and accumulates in the cells. Similar differences in 6-pyruvoyl tetrahydropterin synthase activity have been observed earlier when comparing human and murine liver, resulting similarly in neopterin accumulation only in the human tissue, not in the murine tissue (73). It is clear from Table I, however, that the formation of neopterin by human fibroblasts occurs in addition to tumor necrosis factor-α-triggered tetrahydrobiopterin synthesis, which is comparable in human and murine fibroblasts. On the other hand, the different action of cycloheximide on cytokine-induced pteridine synthesis in human and in murine cells indicates that the cytokine signal may be processed differently in these two species (69).

Impact of cytokines of pteridine concentrations as well as on tetrahydrobiopterin biosynthetic enzyme activities in the human and mouse is summarized in a scheme presented in Figure 4 that is compatible with most of the published results. Cells of the human and mouse, including human macrophages, constitutively contain 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase activities. They are prepared to react to cytokine action with increased activities of GTP-cyclohydrolase I, which then triggers the biosynthesis of tetrahydrobiopterin. This action is not limited to cells of the immune system such as macrophages or lymphocytes, but comprises a number of cytokine-responsive cells, such as fibroblasts, endothelial cells, and tumor cell lines. Due to a lower activity of 6-pyruvoyl tetrahydropterin synthase, neopterin accumulates in addition to tetrahydrobiopterin in human cells, giving rise to the increased neopterin concentrations in diseases challenging cell-mediated immunity.

Functional Roles of Cytokine-Induced Increase of Tetrahydrobiopterin Concentrations

An approach we took to investigate the functional role of cytokine-induced pteridine synthesis was to check for the activities of the pteridine-dependent aromatic amino acid hydroxylases in γ-interferon-treated cells in comparison to untreated cells. We could not

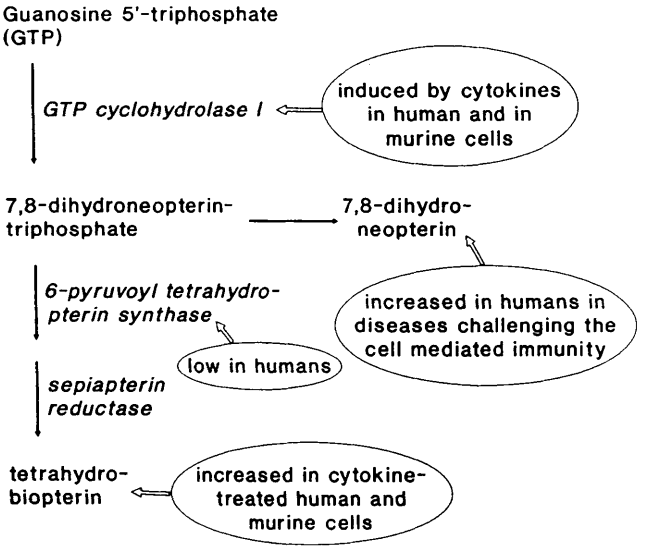

Figure 4. Schematic representation of the influence of cytokines on the biosynthesis of tetrahydrobiopterin in human and in murine cells. For chemical structures of the intermediates, see Figure 2. Among the cytokines tested so far, γ-interferon and tumor necrosis factor-α were the strongest stimuli of pteridine synthesis.

detect, however, activities of phenylalanine 4-hydroxylase, tyrosine 3-hydroxylase, or tryptophan 5-hydroxylase in untreated or γ-interferon-treated cells, which produce a large amount of tetrahydrobiopterin (51). Pilot experiments also did not show evidence for a pteridine-dependent cleavage of ether lipids in the cells (E. R. Werner, G. Werner-Felmayer, F. Snyder, and H. Wachter, unpublished data). Another approach to unravel the role of cytokine-induced tetrahydrobiopterin synthesis was to look for metabolic reactions induced in parallel to pteridine synthesis. In supernatants of stimulated peripheral blood mononuclear cells, a fluorescent peak appearing in parallel to neopterin (46) was identified as 3-hydroxyanthranilic acid (74), a tryptophan metabolite. Subsequent investigations showed that formation of 3-hydroxyanthranilic acid originated from degradation of tryptophan by macrophages (75). Only indoleamine 2,3-dioxygenase is induced by γ-interferon (51); additional enzyme activities are constitutively present in the cells and are unaffected by the interferon treatment (76). Indoleamine 2,3-dioxygenase requires methylene blue and ascorbic acid for full activity (77). The nature of the natural electron donor, however, remains to be determined. Tetrahydrobiopterin was shown to stimulate the indoleamine 2.3-dioxygenase reaction (51, 78, 79). Subsequent experiments with intact cells, however, ruled out a functional role of pteridines for the cleavage of tryptophan: A special case, THP-1 cells treated with phorbol myristate acetate, was found in which kynurenine was formed at a high rate by intact cells without stimulation of pteridine synthesis. In addition, in T 24 cells, depletion of intracellular tetrahydrobiopterin by inhibition of GTP cyclohydrolase I with 2,4-diamino-6-hydroxypyrimidine (80) did not alter the rate of kynurenine formed from tryptophan (81).

In 1989, a new pteridine-dependent enzymatic reaction was discovered: Nitric-oxide synthase in homogenates of activated murine macrophage lines was found to require tetrahydrobiopterin for full activity (82, 83). Tetrahydrobiopterin-dependent synthesis of nitric oxide was also seen in homogenates of cytokine-treated smooth muscle cells (84) and in rat renal mesangial cells (85). All these studies were performed with cell homogenates. We then decided to use a similar approach as we had used for testing the relevance to pteridines for the indoleamine 2,3-dioxygenase reaction (81). In intact murine fibroblasts, inhibition of cytokine-induced biosynthesis of tetrahydrobiopterin by the GTP cyclohydrolase I inhibitor 2,4-diamino-6-hydroxypyrimidine inhibited the formation of nitrite and nitrate from L-arginine (86). Restoration of the effect of the inhibitor in the presence of sepiapterin, which restores tetrahydrobiopterin concentrations via a salvage pathway (13), and impact of methotrexate, which inhibits the reduction of 7,8-dihydrobiopterin to the tetrahydroform, demonstrated that the effect is specific for tetrahydrobiopterin (86). Thus, in contrast to indoleamine 2,3-dioxygenase, nitric-oxide synthase activity is limited by intracellular tetrahydrobiopterin concentrations. These results suggested that a major role of cytokine-induced pteridine synthesis is to provide cells with the active tetrahydrobiopterin cofactor required for the nitric-oxide synthase reaction (86). In a similar approach, the importance of intracellular tetrahydrobiopterin for cytokine-induced nitric oxide formation was shown in murine brain endothelial cells (87) and in rat vascular smooth muscle cells (88), as well as for the constitutive, calcium-dependent, short-term nitric oxide synthesis in porcine aortic endothelial cells (89).

Nitric-oxide synthase occurs in at least three different isoforms (reviewed in 90, 91). Calcium/calmodulin-dependent isoforms from brain (92) and endothelial cells (93, 94) and a calcium-independent, cytokine-inducible form (95–97) have been cloned. Recent results suggest that the cytokine-inducible form contains calmodulin as a subunit (98). Nitric-oxide synthase induced by LPS in rat liver was stimulated by calmodulin, but not by calcium (99). Whereas only the cytokine-inducible type of nitric-oxide synthase was initially thought to require tetrahydrobiopterin, Mayer and co-workers (100) first demonstrated that calcium/calmodulin-dependent nitric-oxide synthase purified from porcine cerebellum is also stimulated by tetrahydrobiopterin. Subsequently, tetrahydrobiopterin stimulation was shown for nitric-oxide synthase purified from murine macrophages (101, 102), rat cerebellum (103), rat macrophages (104), rat polymorphonuclear neutro-

phils (105), nonadrenergic, noncholinergic nerve-containing tissue (106), bovine (107) and human brain (108, 109), bovine endothelial cells (110), and liver of endotoxin-treated rats (99). The initial finding of tetrahydrobiopterin independence of nitric-oxide synthase from brain was based on the observation that the pure enzyme shows activity without external addition of the cofactor. Analysis of enzyme purified from porcine cerebellum then demonstrated that tetrahydrobiopterin remains bound to the enzyme (111). Thus, enzyme activity in absence of exogenously added tetrahydrobiopterin is also compatible with the hypothesis of an absolute requirement of nitric-oxide synthase for this cofactor. Tetrahydrobiopterin was then also found in preparations of rat cerebellar nitric-oxide synthase (112) and the bovine endothelial cell enzyme (J. S. Pollock, E. R. Werner, J. A. Mitchell, U. Förstermann, and F. Murad, unpublished). The activity of macrophage nitric-oxide synthase correlated to the amount of enzyme-bound tetrahydrobiopterin, with a maximum at one bound tetrahydrobiopterin per 130-kDa subunit (113). Only tetrahydrobiopterin, but not neopterin, derivatives copurified with nitric-oxide synthase from human cerebellum (109).

The molecular mechanism of the stimulation of the nitric-oxide synthase reaction by tetrahydrobiopterin remains to be demonstrated. One remarkable feature is the strict requirement of nitric-oxide synthase for the naturally occurring stereoisomer of tetrahydrobiopterin, which has the 6-R conformation. Other tetrahydropteridines, including 6-S tetrahydrobiopterin, had almost no stimulatory effect on the nitric-oxide synthase reaction in material derived from rodent (83, 103), porcine (B. Mayer, M. John, and E. Böhme, unpublished), or human (109, 114) tissue. Although the chirality of C6 is also important for pteridine-dependent aromatic amino acid hydroxylases (115), the difference is much less pronounced and stimulation of enzyme activities can be observed with several tetrahydropterins. The concentration for half maximal stimulation of nitric-oxide synthase is remarkably low at 0.4 μM (100), and a saturating effect is seen at concentrations of 1 μM. NADPH is consumed in the reaction in stoichiometric amounts; a total of 1.5 moles are required per mole of citrulline formed (111, 116). One mole of tetrahydrobiopterin, in contrast, can catalyze the formation of at least 10 moles of citrulline (103, 111). Giovanelli *et al.* (103) could not find evidence for a redox reaction of tetrahydrobiopterin during the synthesis of nitric oxide from L-arginine, and suggested that an allosteric effect is responsible for the stimulation. In addition, no detectable 7-biopterin derivatives are formed in the nitric oxide synthase reaction (E. R. Werner, B. Mayer, and H. Wachter, unpublished). The 7-isomer of biopterin has been detected in phenylalanine hydroxylase incubation mixtures in the absence of pterin 4a-hydroxydehydratase (117, 118). This enzyme accelerates conversion of the 4a-hydroxy derivative, the initial product formed from tetrahydrobiopterin by the hydroxylation step, to the quinonoid 5,6-dihydrobiopterin, which is then recycled by dihydropteridine reductase to the active tetrahydrobiopterin cofactor. Although these findings argue against a role of tetrahydrobiopterin as redox cofactor in the nitric-oxide synthase reaction, a recycling of the tightly bound cofactor cannot be excluded on the basis of these experiments.

Another approach to unravel the role of tetrahydrobiopterin in the nitric-oxide synthase reaction was to find out in which step of this complex reaction tetrahydrobiopterin is involved. It was tempting to assume that this cofactor is required in a first step that was suggested to be a hydroxylation of arginine on a terminal guinidinonitrogen (82, 111). Experiments with *N*-hydroxyarginine, however, indicated that tetrahydrobiopterin is also necessary for the further conversion of this assumed intermediate to citrulline (116, 119). Thus, tetrahydrobiopterin appears to be required for the whole nitric-oxide synthase reaction. One example of a pteridine-independent step of nitric-oxide synthase is the uncoupled, calcium/calmodulin-dependent formation of hydrogen peroxide, which occurs in the absence of L-arginine or tetrahydrobiopterin (120).

A question open for some time was the significance of tetrahydrobiopterin-dependent nitric oxide formation in humans. Since nitric oxide synthase could not be induced in human cells *in vitro* by protocols successful in murine cells (discussed in 121), the occurrence of an inducible nitric oxide synthase in humans was speculative. Recent evidence from *in vitro* (122) as well as *in vivo* (123, 124) observations confirm the stimulation of nitric oxide synthesis in humans by cytokines. We showed recently for human umbilical vein endothelial cells that the constitutive nitric-oxide synthase can be influenced by manipulation of intracellular tetrahydrobiopterin levels. Depletion of cells by inhibiting *de novo* synthesis of tetrahydrobiopterin with the GTP cyclohydrolase I inhibitor 2,4-diamino-6-hydroxypyrimidine suppressed nitric oxide formation. Increasing intracellular tetrahydrobiopterin concentrations by treating cells with sepiapterin or by stimulating *de novo* synthesis with cytokines resulted in increased formation of nitric oxide (68). We could also recently show that the human cervix carcinoma cell line ME-180 expresses brain type nitric oxide activity (114). Similar to human umbilical vein endothelial cells, cytokines lead to increased nitric oxide formation by increasing the intracellular tetrahydrobiopterin concentrations in this cell line. These results clearly indicate that cytokine-induced tetrahydrobiopterin synthesis can stimulate the constitutive nitric oxide synthase in human cells, which suggests that the enzyme is not

saturated with the cofactor in these *in vitro* model systems.

In addition to the new role in the nitric oxide reaction, tetrahydrobiopterin was found to be functionally connected to the proliferation of murine erythroleukemia cells (125, 126). In two human cell lines, however, severe deletion of intracellular tetrahydrobiopterin did not have any effect on cell proliferation (127). Tetrahydrobiopterin was shown to stimulate proliferation of human peripheral blood mononuclear cells at suboptimal doses of interleukin 2 (128). An increase of the affinity of the interleukin 2 receptor on a human T cell line was seen only with 6-R tetrahydrobiopterin, but not with other pteridines (129). Another new role for tetrahydrobiopterin was found to be the release of acetylcholine (130), dopamine, serotonin, and glutamate (131) by tetrahydrobiopterin in brain. Nitric-oxide synthase is found in many brain regions (92) as well as in interleukin 2-stimulated lymphocytes (132). The observed effects and nitric-oxide synthase share an unusual selectivity for the 6-R-isomer of tetrahydrobiopterin. It will be interesting, therefore, to learn whether some of these new roles are mediated by nitric oxide.

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