Review

Biosynthesis and function of tetrahydrobiopterin

David S. Duch and Gary K. Smith

Division of Cell Biology, Wellcome Research Laboratories, Research Triangle Park, NC, USA

Tetrahydrobiopterin (BH_4) belongs to the class of pteridines (fused pyrimidopyrazines) possessing a 2-amino-4-oxo substitution of the pyrimidine moiety. BH_4 is the coenzyme for tyrosine, tryptophan, and phenylalanine hydroxylases; for the glycerol ether monooxygenase; and probably for the arginine utilizing nitric oxide synthase in rodent macrophages. The function of BH_4 in these reactions derives from the ability of the cofactor to react directly with molecular oxygen to form an active oxygen intermediate that can hydroxylate substrate. In the hydroxylation process, the coenzyme loses two electrons and is regenerated in vivo in an NADH-dependent reduction catalyzed by DHPR.

This review of BH_4 describes studies on biosynthesis, analysis, and the role of pterins in the immune response and in some diseases reported since our previous review.[1] For further general and more detailed information on BH_4 and other pterins, the reader is referred to the monograph series Chemistry and Biology of Pteridines *and the three-volume set* Folates and Pterins.

Keywords: biosynthesis; pterins, immunity; pterins, disease states

Introduction

BH_4 Biosynthesis

Pathway. BH_4 is synthesized in mammals and other eucaryotes in the tissues where it is used. In the first half of the 1980s, it became apparent that the BH_4 biosynthetic pathway that had been published was incorrect[1-4]; the pathway did not involve dihydrofolate reducatase, and all of the pterin intermediates beyond NTP were H_4pterins rather than H_2pterins. The current, generally accepted pathway is shown in *Figure 1*.

The commiting step in BH_4 biosynthesis is the conversion of GTP to NTP and formate by the enzyme GTP-CH.[1-2] The mechanism of the reaction has not been studied extensively and none of the proposed intermediates have been isolated. The conversion of NTP to PTP, the first H_4pterin intermediate, is catalyzed by pyruvoyl H_4pterin synthase (PTP synthase).[5-9] This reaction proceeds in the absence of an external reducing agent, and requires only Mg^{2+} as a cofactor. The mechanism is inferred by the observations that the C-6 proton derives exclusively from H_2O[10,11] and the C-3′ proton derives at least partially from H_2O as well.[11] The transfer of less than one proton from H_2O to the C-3′ suggests that either a multivalent base on the enzyme is involved or that this base only partially equilibrates with solvent during proton abstraction and donation. Further, there is no significant transfer of the 1′ or 2′ hydrogen to either C-6 or 3′ since unlabelled BH_4 is synthesized from NTP tritiated in both the 1′ and 2′ positions.[12] It is unknown whether triphosphate elimination or H_4pterin formation occurs first. The structure of PTP has been determined by its electrochemical characteristics, UV/VIS spectrum, ^{1}H-NMR spectrum, and by chemical means.[5-9,13]

Sepiapterin reductase (SR) is capable of catalyzing the NADPH-dependent reduction of both side chain keto groups of PTP to produce BH_4 with the proper stereochemistry.[8,9,14,15] The reduction of PTP to BH_4 in crude extracts is inhibited by the SR inhibitor NAS and by specific antibodies to the enzyme.[14,16] Thus, SR catalyzes the reductions in these extracts. The primary isolatable intermediate in the reduction of PTP by SR is 1′-hydroxy-2′-oxy-H_4pterin[14]; 1′-hydroxy-2′-oxo-H_4pterin is also the compound produced in the reverse reaction from BH_4.[17] These data indicate that

Address reprint requests to Dr. Duch at the Division of Cell Biology, Wellcome Research Laboratories, Research Triangle Park, NC 27709, USA.
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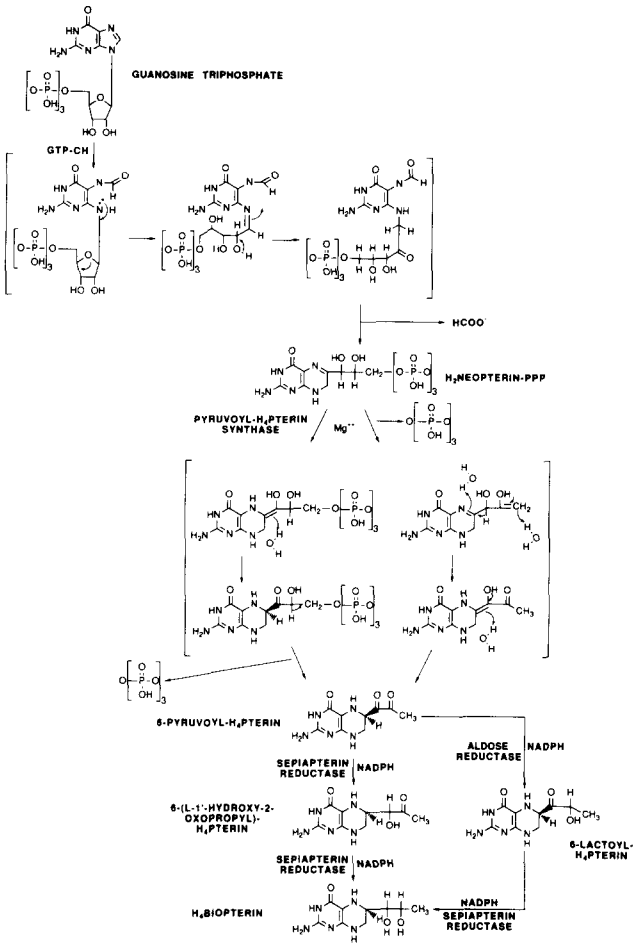

Figure 1 The BH_4 biosynthetic pathway

the enzyme preferentially reduces the 1′ keto group which is consistent with the high activity of the enzyme toward sepiapterin and lactoyl-H_4pterin compared to the 1′-hydroxy-2′-oxo-H_4pterin.[14] The structure of 1′-hydroxy-2′-oxo-H_4pterin has been determined by chemical means, and the 1′ stereochemistry has been shown to be that of BH_4.[6,9,17] This compound is produced in crude extracts from both NTP and PTP; its synthesis is optimized by the addition of low concentrations of the SR inhibitor NAS.[9,14] The production of 1′-hydroxy-2′-oxo-H_4pterin in crude extracts is inhibited by high concentrations of NAS and by the SR antibody,[14,16] indicating that SR is responsible for the synthesis of this compound. SR can catalyze the reduction of 1′-hydroxy-2′-oxo-H_4pterin to BH_4 and, since low concentrations of NAS in crude extracts enhance the accumulation of 1′-hydroxy-2′-oxo-H_4pterin to BH_4, SR presumably is the enzyme responsible for catalyzing its reduction by BH_4 in vivo.[9,14] This demonstrates that GTP-CH, PTP synthase, and SR are the necessary and sufficient enzymes required for the conversion of GTP to BH_4, as shown in *Figure 1*.

Lactoyl-H_4pterin, observed in extracts under some circumstances,[5,8,9,14,16] has also been proposed as a biosynthetic intermediate to BH_4.[18] Although SR is capable of catalyzing the reduction of lactoyl-H_4pterin to BH_4,[5,14,18,19] neither NAS nor SR antibodies inhibit its production, indicating that SR is not responsible for the synthesis of lactoyl-H_4pterin.[14,16] Rather, another reductase, initially termed PTP reductase,[20,21] but now known to be aldose reductase, catalyzes its synthesis.[22-23] It is not known to what extent this second reduction sequence PTP > lactoyl-H_4pterin > BH_4 is used in individual tissue types. However, several lines of evidence suggest that if SR and aldose reductase are truly soluble in the cytosol, and as such are accessible to PTP, the reduction sequence of the 1′ and 2′ ketones of PTP may be tissue dependent (23, Smith unpublished). An antibody to aldose reductase has been produced that inhibits PTP reductase but not SR. In crude rat brain extracts, under conditions in which the antibody totally inhibited PTP reductase, BH_4 biosynthesis was inhibited no more than 60%.[23] This suggests that the reaction sequence catalyzed by aldose reductase accounts for approximately half of the BH_4 biosynthesis flux in rat brain and that reduction of the 1′ and 2′ keto groups proceeds at approximately equal rates. In a similar study with rat liver, the antibody was without effect on BH_4 biosynthesis,[23] indicating that aldose reductase is not important for BH_4 biosynthesis in liver. This is consistent with the low levels of aldose reductase found in Western blots of liver relative to several brain regions.[23] We have measured the accumulation of lactoyl-H_4pterin from NTP either in the absence of NAS or in the presence of 100 μM NAS, enough for complete inhibition of SR and BH_4 biosynthesis. Both rat liver and rat reticulocytes synthesized 1′-hydroxy-2′-oxo-H_4pterin, while neither synthesized detectable lactoyl-H_4pterin. Other rat tissues synthesized lactoyl-H_4pterin in the relative amounts of kidney > testes > adrenal > brain (14, Smith unpublished observations). These results indicate that lactoyl-H_4pterin is not a biosynthetic intermediate in rat reticulocytes or liver but may be in some other tissues.

Biosynthetic enzymes

GTP Cyclohydrolase. This enzyme catalyzes the conversion of GTP to NTP,[2] and its distribution closely parallels that of BH_4.[1] The enzyme has been purified from both procaryotes[24-26] and eucaryotes.[27-31] The *E. coli* enzyme, which is used in tetrahydrofolate rather than BH_4 synthesis, has a molecular mass (M_r) of 210 kDa and subunits of 25.5 kDa[24]; it has been cloned and crystallized.[26] Homogeneous or nearly homogeneous eucaryotic GTP-CH has been prepared from *Drosophila*, rat liver, and human liver.[27-31] The *Drosophila* enzyme is 575 kDa with subunits of 39 kDa[27]; the rat liver enzyme is 300 kDa with subunits of 30 kDa[31]; and the human enzyme is 440-453 kDa with subunits reported as 50 kDa or 140 kDa.[29,30] The purified enzyme is unstable.[27-31]

Antibodies to the human[29] and *E. coli*[26] enzymes have been produced. Inhibitors of GTP-CH have been reported.[32-35] The most useful for depleting BH_4 in

whole cells or animals has been 2,4-diamino-6-hydroxypyrimidine.[36,54,56] The *Drosophila* enzyme is encoded by the Punch gene.[37] cDNA clones have now been obtained and are being used to investigate regulation of the enzyme at the genetic level.[28] The biochemical defect of hph-1 mouse mutant is expression of low levels of the enzyme.[38]

Pyruvoyl H_4pterin synthase. This enzyme converts NTP to PTP and has Mg^{2+} as cofactor. Chemical synthesis of the substrate has been reported.[39] PTP synthase has been purified to apparent homogeneity from *Drosophila*,[40] salmon liver,[41] and human liver.[42] M_r of the purified *Drosophila* enzyme is 83 kDa with 37.5 kDa subunits; M_r of the salmon liver enzyme is 68 kDa with 16 and 17 kDa subunits; and the M_r of the human liver enzyme is 83 kDa with 19 kDa subunits.[40-42] Substrate K_m has been reported to be 10 μM for a partially purified *Drosophila* preparation[43] and 100 μM for the homogeneous enzyme[40]; 2.2 μM for the salmon liver enzyme[41]; and 10 μM for the human liver enzyme.[42] Antibodies have been produced against the enzymes from *Drosophila* and salmon.[40,41]

Aldose reductase. Aldose reductase catalyzes the NADPH-dependent reduction of a variety of a-hydroxyketones including lactoyl-H_4pterin.[22,23,44-47] For a detailed discussion of this enzyme, the reader is referred to recent reviews.[44-48] The substrate specificity of the enzyme is quite broad,[22,23,44-47] and its tissue distribution closely parallels the distribution of lactoyl-H_4pterin synthesis discussed above (46, Smith, unpublished). Using an immunoassay, kidney was found to possess three times as much aldose reductase as brain; liver and erythrocytes were found to have at least 10-fold less than brain. Highest levels were found in seminal vesicle, skeletal muscle, and lens, and were 5–8 times higher than in kidney. The distribution of the enzyme does not parallel overall BH_4 biosynthesis.[1] M_r of the rat brain and human liver enzymes are 37 kDa and 35 kDa, respectively.[20,21] Transfer of the pro R-hydrogen of NADPH to PTP has been demonstrated for the human liver enzyme.[21] The K_m for PTP is 1.8 μM with the human enzyme and 2 μM with the rat enzyme.[20,21] The K_m for NADPH with the human enzyme is 5.5 μM when PTP is the substrate and 5 μM when typical aldose reductase substrates are used.[21,44-46] A variety of aldose reductase inhibitors, which should be of use in the study of the role of this enzyme in BH_4 biosynthesis, have been reported.[48] Antibodies have been prepared against the enzyme from rodent and human sources.[22,23]

Sepiapterin reductase. This enzyme catalyzes the NADPH-dependent reduction of a variety of pterin and non-pterin ketones.[14,49] SR is typically purified from rat erythrocytes.[50] Distribution of the activity is quite broad and does not parallel BH_4 biosynthesis, but is present in all tissues that make the cofactor.[1] M_r of the rat erythrocyte enzyme is 56 kDa with 28 kDa subunits.[50] Antibodies to the rat enzyme have been produced.[14,16] The complete amino acid and nucleotide sequences of the rat enzyme have been reported.[51,52] K_m values for the erythrocyte enzyme for PTP, 1′-hydroxy-2′-oxo-H_4pterin, lactoyl-H_4pterin, and sepiapterin are 2,7,8,5–14 μM, respectively.[14,49,50] Based upon V/K, PTP is the best pterin substrate for the enzyme.[14]

The first potent inhibitor reported for the enzyme was N-acetylserotonin, which has a K_i = 0.1–0.2 μM[53]. More recently, N-acetyldopamine, K_i = 0.4 μM, and N-acetyl-m-tyramine, K_i = 0.1 μM, were found to have affinities similar to that of N-acetylserotonin.[54] Chloroacetylserotonin, K_i = 0.006 μM, and methoxyacetylserotonin, K_i = 0.008 μM, recently were reported and are now the tightest binding inhibitors known.[54] The inhibitors are competitive with the pterin substrate and have been used in studies on the functions of BH_4[54,55] and flux through the biosynthetic pathway.[56]

SR catalyzes the isomerization of lactoyl-H_4pterin to 1′-hydroxy-2′-oxo-H_4pterin; isomerase activity is stimulated by low concentrations of NADP or NADPH.[19,57] These observations led to the suggestion that this isomerase activity is important in vivo in the reduction of lactoyl-H_4pterin and PTP to BH_4.[19] However, the rate of lactoyl-H_4pterin reduction by SR was found to be at least 20-fold faster than the rate of lactoyl-H_4pterin isomerization to 1′-hydroxy-2′-oxo-H_4pterin.[19] This suggests that the isomerase activity is not kinetically competent in the reduction. In addition, lactoyl-H_4pterin is a better substrate for SR-catalyzed reduction than is 1′-hydroxy-2′-oxo-H_4pterin,[14] which also suggests that isomerization of lactoyl-H_4pterin to 1′-hydroxy-2′-oxo-H_4pterin during the reduction of the former is unlikely. Further work on the relevance of this isomerase activity to SR-catalyzed reduction of pterin and non-pterin substrates is needed.

Regulation of biosynthesis. Reviews on regulation of BH_4 biosynthesis have been published,[1,58,59] and the reader is directed to them for a discussion of earlier work. In at least some tissues and cells in culture, BH_4 levels regulate the flux through BH_4-dependent hydroxylation reactions.[1,58,59] These levels of BH_4 are, at least in part, controlled by the rate of de novo biosynthesis.[1,58-64]

Mammalian BH_4 biosynthesis is regulated largely through changes in GTP-CH levels. In the adrenal medulla, BH_4 is required for tyrosine hydroxylation in catecholamine synthesis, and BH_4 levels are regulated by the splanchnic nerve through GTP-CH.[58] The mechanism of this regulation has been elucidated in adrenal medullary cells in culture. Increases in cyclic AMP produced following agonist binding to nicotinic receptors cause increases in GTP-CH levels and, as a result, BH_4 levels.[61] Since these increases are inhibited by cycloheximide, the GTP-CH increase is likely due to new protein synthesis. Catecholamine depleting agents also cause an increase in adrenal medullary GTP-CH levels and in most cases an increase in BH_4 levels as well.[58,61,65] These changes do not appear to

be cyclic AMP mediated,[61] but new protein synthesis is required.[58,65] In the pheochromocytoma clone PC12h, BH_4 levels are increased transiently by nerve growth factor.[66] Elevation of cyclic AMP in the cells can mimic the effect, but it is not known whether GTP-CH is involved.

Although the role of BH_4 in the adrenal cortex is unknown, the cofactor is produced and regulated in this tissue.[58] In vivo, both insulin and reserpine produce protein synthesis dependent increases in adrenal cortical GTP-CH which are controlled by ACTH released from the pituitary.[67] Both insulin and reserpine cause the pituitary to release ACTH, hypophysectomy prevents the GTP-CH increases, and ACTH can reverse the effect to hypophysectomy. ACTH also can produce cyclic AMP dependent increases in GTP-CH and BH_4 levels in the Y-1 adrenal cortical tumor line in culture.[68] ACTH does not appear to have a regulatory effect on GTP-CH in the adrenal medulla, liver, bone marrow or spleen.[1,58,61]

The regulation of BH_4 biosynthesis in blood cells is interesting because it provides an indication of intracellular localization of GTP-CH (red cells) and provides a very useful assay of immune system (macrophage) activation.[29,69-71] BH_4 is synthesized in reticulocytes but not erythrocytes.[72] The only BH_4 biosynthetic enzyme lost in the last step of red cell maturation is GTP-CH (72, Smith and R. Mullin, unpublished). An immunoenzymatic method has been used to show the presence of GTP cyclohydrolase in the reticulated cytoplasmic structure of reticulocytes and the complete absence in mature erythrocytes.[73] Since this suggests that the enzyme is associated with the endoplasmic reticulum,[72] it is likely extruded on the endoplasmic reticulum during maturation. BH_4 biosynthesis in bone marrow, spleen, erythrocytes, and reticulocytes has also been studied in mice with chemically induced and genetically conditioned reticulocytosis and similar results were obtained.[74]

In macrophages, GTP-CH is regulated by external stimuli since IFN-γ can induce the enzyme.[71] However, this induction results in the synthesis of NTP alone and not BH_4, which is not produced in human macrophages.[69-71] Thus, in these cells, the lack of BH_4 biosynthesis is not solely due to the loss of GTP-CH, but also reflects the absence of PTP synthase and the resultant lack of conversion of NTP to PTP.[70,71] Thus, while BH_4 synthesis is generally controlled by GTP-CH, the results in macrophages demonstrate that PTP synthase deficiency also controls BH_4 biosynthesis in some cells. Rodent macrophages are not deficient in PTP synthase and as such do produce BH_4.[70,75]

Rat liver sepiapterin reductase is stimulated to 150% of control by glucagon, but this treatment has no significant effect upon either GTP-CH or BH_4 levels, indicating that at least in this case changes in SR levels have little control over BH_4 levels.[76,77] Similarly, inhibition of SR in several cell lines in culture has required very high levels of SR inhibitors to produce inhibition of BH_4 biosynthesis.[54-56] This also suggests that SR has little control over the rate of BH_4 biosynthesis.

Analysis and distribution of unconjugated pterins

Unconjugated pterins exist in vivo as the tetrahydro, dihydro and fully oxidized species. The majority of assays for pterins described in our previous review[1] were procedures for the measurement of the fully oxidized species produced upon oxidation of the reduced forms, either specifically or nonspecifically. These assays included both HPLC, usually with fluorescence detection, and RIA methods. Since BH_4 and tetrahydroneopterin decompose to pterin upon base oxidation and to fully oxidized biopterin or neopterin upon acid oxidation, the assays could be used to determine the levels of these compounds as well as their oxidation states.

The introduction of HPLC coupled with electrochemical detection made the direct determination of the reduced pterins possible. Hyland and coworkers[78,79] described a method using reversed phase HPLC for the analysis of all three species of biopterin as well as other pterins in a single chromatographic run. Pterins of all three oxidation states were detected using sequential coulometric, electrochemical, and fluorescence detection. Tetrahydropterins were measured electrochemically, dihydropterins by fluorescence following post-column electrochemical oxidation, and oxidized pterins by their natural fluorescence. A similar method was also described by Powers et al.[80]

Increased sensitivity and/or decreased sample preparation were achieved using immunoassays; radioimmuno-, enzyme-linked immunosorbent and polarization fluoroimmunoassays have been described.[81-83] Other reported methods of HPLC analysis represent modifications of existing methods.[84-86] A more comprehensive review of pterin analysis has been published by Hyland and Howells.[87]

Unconjugated pterins are found in most mammalian tissues and fluids, with levels in the pineal being as high as 12.5 μg/g.[1,88] In addition, unconjugated pterins have been found in cerebrospinal fluid,[89] saliva,[90] milk from several mammalian species,[91] amniotic fluid,[92] and mammalian ocular tissue.[93,94] Neopterin and biopterin levels in amniotic fluid were highest in late gestation and were substantially higher than the levels found in maternal serum during this period. BH_4 levels, as well as the biosynthetic enzymes, were decreased in human senile cataracts relative to age-matched clear human lens. High levels of BH_4 were found in rat reticulocytes, which decreased with maturation of these cells to erythrocytes. These levels reflect the loss of GTP-CH upon maturation.[73]

Dhondt et al.[95] described a patient with mild hyperphenylalaninemia and with an unknown pterin-like compound in the urine and CNS. Blaskovics and Giudici[96] also described a patient having similar symptoms. Subsequent studies[97,98] led to the isolation and characterization of three new 7-substituted pterins; L-erythro-7-iso-biopterin (primapterin), D-erythro-7-iso-neopterin (anapterin) and L-erythro-6-oxo-7-iso-

biopterin (6-oxo-primapterin). Primapterin and anapterin were found in low levels in human liver, saliva, and urine, and were elevated in the patients described above. However, 6-oxo-primapterin could not be detected in normal individuals.[90,97,98] The ratio of biopterin to primapterin in the patients' urines was 1:1 and levels of primapterin rose in parallel to biopterin following a loading dose of BH_4. The formation of these 7-substituted pterins was reported to be dependent on the metabolic loss of the dehydratase enzyme required for BH_4 recycling.[99]

Pterins and the immune system

Changes in pterin levels under conditions that result in activation of the immune response led to the suggestion that reduced pterins may have some role in the immune response.[1] However, when the role of pteridines in the functioning of the immune system was studied in a patient with a GTP cyclohydrolase deficiency,[100-102] no significant deficiencies in cellular and humoral immunity could be found. Thus, the importance of pterins in the immune response remains uncertain.

A number of studies designed to investigate the role of pterins in this process have been reported. BH_4 biosynthesis was studied in mutant mice with immunological defects[103] in an attempt to select in vivo model systems. Ziegler and coworkers[104,105] measured changes in pterin synthesis in unfractionated cultures of cells obtained from murine spleen or human pbmc stimulated by lectin. In murine cells, there was an increase in cellular biopterin and other pterins, but not neopterin, which preceded DNA synthesis. In human pbmc, there were increases in both biopterin and neopterin.

Monocytes and macrophages. Schoedon et al.[70,75,106] studied pterin distribution in fractionated human pbmc. Only neopterin was found in human monocytes since PTP synthase was lacking in these cells. GTP-CH and neopterin levels increased rapidly following treatment with supernatants from lectin or MLC-stimulated T cells. In contrast, BH_4 was the only pterin found in murine macrophages. Huber et al.[107] demonstrated that immune responses in humans were accompanied by an increased release of urinary neopterin. They also demonstrated that monocytes stimulated with supernatants from activated T cells released large amounts of neopterin into the culture medium. The T-cell-derived factor responsible for this is IFN-γ.[107,108] Increased levels of urinary neopterin were also found in patients treated with TNF-α[109] and IL-2.[110] In both cases, the effect was most likely mediated through increased IFN-γ production. GTP, GTP-CH, and neopterin levels all significantly increased in human monocytes treated with supernatants from activated T cells, IFN-γ, or IFN-α.[70,106,111-113] No detectable levels of biopterin were found, and the data indicates that monocytes have lost the ability to synthesize BH_4 due to the lack of PTP synthase. In contrast, murine macrophages, which possess PTP synthase, synthesize BH_4, but no neopterin can be detected in these cells.[70]

T Lymphocytes. Schoedon et al.[70,75,106] reported that T cells had low concentrations of neopterin, biopterin, and pterin and low levels of GTP-CH, which increased significantly in stimulated cells. There were also low but measurable levels of PPH_4S and high levels of SR which did not change following lectin treatment.

Clonal expansion of T lymphocytes is mediated by IL-2, a T cell derived lymphokine synthesized and secreted following stimulation by mitogens or antigens.[114] Ziegler et al.[115] postulated that BH_4 modulates the activity of IL-2. BH_4 increased DNA synthesis in human pbmc[115] and in IL-2 receptor-positive T cells[116] treated with IL-2; this effect was dependent on the concentration of both BH_4 and IL-2. A transient rise in neopterin and biopterin was also observed. Similar transient effects were found when IL-2 receptor-positive T cells as well as several established cell lines were treated with phorbol ester.[117] In subsequent studies using the IL-2 dependent cloned murine cytotoxic T lymphocyte line CTLL-2, BH_4 increased the rate of internalization of IL-2 and it was postulated that BH_4 participates in the control of IL-2 receptor assembly.[18] Scatchard analysis indicated that BH_4 increased the affinity of IL-2 for the receptor and decreased the number of binding sites. The dissociation rate constantly decreased to 50 percent of control values in the presence of BH_4 and the half-time for dissociation increased twofold.

Other studies indicate that IFN-γ may also be involved in the synthesis of biopterin in T cells as well as monocytes. IFN-γ triggered renewed biopterin synthesis and release in T cells 2–4 days after their stimulation by lectin, which could be neutralized by anti-IFN-γ antibody.[105] HTLV-1 transformed $CD4^+$ T cell lines were also used[119] to study the effects of IL-2 and IFN-γ on BH_4 biosynthesis. The synthesis of BH_4 as well as the biosynthetic enzymes GTP-CH and PTP synthase is increased by treatment of cells with IFN-γ and is further enhanced by co-addition of IL-2. SR is only transiently increased by this combination; IL-2 alone has no effect. These results are different from those obtained by the same laboratories in lectin-stimulated T cells.[120] PHA produced an increase in GTP-CH that was maximal at 48 hr and then declined; SR showed a continued increase over the entire period and PPH_4S remained unchanged. These contrasting results raise questions regarding the use of these cultured cell lines as models of peripheral blood mononuclear cells.

Cultured cell lines. Cultured cells also have been studied as possible models for both T cells and monocytes. MOLT-4 cells were found to contain GTP-CH, neopterin and biopterin at levels comparable to those found in stimulated human T cells.[70] However, these cells did not respond to IFN-γ, lectin, or MLC. Biopterin biosynthesis was also studied in the murine T cell lines

OVA-T, which is dependent on IL-2 for growth, and EL-4, which grows independently of IL-2.[70] EL-4 had low levels of biopterin and did not respond to IL-2 with increased synthesis. Biopterin levels increased fourfold following the addition of IL-2 to OVA-T cells. With regard to monocytic cells lines, no pterin synthesis was found in HL-60 or U-937 cells.[70] However, when U-937 was cloned, several of the clones, but not the parent line, responded to IFN-γ with neopterin synthesis.[121] The human myelomonocytic cell line THP-1 was shown to have properties similar to freshly purified human monocytes.[122,123] These cells had an intracellular pterin pattern similar to monocytes and released neopterin upon stimulation with IFN-γ. In THP-1, as in monocytes, IFN-γ induced parallel induction of neopterin release and tryptophan cleavage by indoleamine dioxygenase and was potentiated by TNF-α, LPS and dexamethasone. IFN-α and IFN-β also induced these pathways in both cell lines, but to a much smaller degree. IFN-γ also induced both tryptophan cleavage and pterin biosynthesis in other permanent cell lines, but the pattern of induction was different than that seen in monocytes THP-1.[123,124]

Nitric oxide formation in macrophages. A role for BH_4 in the formation of nitric oxide, nitrite and nitrate from arginine in macrophages has been proposed. Studies showed that nitric oxide formation from arginine in murine macrophages and murine macrophage cell lines could be induced by IFN-γ, LPS, or a combination of the two.[125-127] Using the end-products nitrite and nitrate as an indicator of synthetic activity, Stuehr et al.[128] found that the enzymatic system in activated murine macrophages responsible for the synthesis of nitrogen oxides from arginine is cytosolic and consists of at least one inducible and two constitutive components, one a low and the other a high molecular weight fraction. Using extracts from the murine cell line, RAW 264, the low molecular weight component, was identified as BH_4.[129] Half-maximal velocity required 20–30 nM BH_4. BH_2 could substitute for BH_4, but studies using BH_2 and dihydrofolate reductase inhibitors indicated that BH_2 must be converted to BH_4 to be active. Similar results were obtained by Tayeh and Marletta[130,131]; they also found that stoichiometric amounts of NADPH relative to BH_4 are required for arginine consumption and optimal product formation. The proposed mechanism of action consistent with the data includes N-hydroxylation of arginine as the initial step. However, since BH_4 is not synthesized in human macrophages, the relevance of this mechanism in humans remains questionable.

Pterins and disease states

Neopterin. The demonstration that neopterin levels increase following stimulation of cellular immunity associated with increased macrophage activity led to a large number of studies that used neopterin release as a marker for disease states.[107] No attempt will be made to discuss all these reports, but reviews can be found elsewhere.[1,132,133] Elevated neopterin levels were demonstrated in allograft rejection; in viral, bacterial, and protozoal infections; in autoimmune diseases such as rheumatoid arthritis, ulcerative colitis, and Crohn's disease; and in neoplastic disease.

In a number of studies, there was a positive correlation between urinary neopterin levels and other clinically used indices of disease status.[134-141] These observations led to the suggestion that measurement of urinary neopterin levels could be used to monitor disease activity. However, despite these correlations, the value of urinary neopterin levels as a clinical marker is still questionable. Neopterin levels can rise in response to common and clinically unimportant infections, and in some cases levels can exceed those due to the disease being studied. The wide range of conditions that can cause elevated neopterin levels could result in false positives and thus casts doubt on the value of this parameter for measuring changes in a specific disease state.

BH_4 in neurological diseases. A variety of neurological disease states, including Parkinson's disease, Alzheimer's disease, dystonia, and depression, present with decreased levels of BH_4 in brain tissue and CSF. The initial reports have been reviewed.[1,142-144]

Loss of dopamine producing neurons is responsible for some of the symptoms of Parkinson's disease. Because BH_4 is produced in the same cells that produce dopamine, it is reasonable that loss of dopamine-containing cells should produce a decrease in CNS BH_4 levels.[1,142-144] Indeed, decreased levels of BH_4 have been reported, and the extent of BH_4 decrease has been correlated with the extent of disease.[1,142-146] The observed depletion of BH_4 combined with reports that BH_4 levels regulate the rate of hydroxylation, led to BH_4 replacement therapy for this disease. However, for both early and late stage Parkinson's disease, BH_4 replacement has been largely ineffective.[1,142-144,147,148] Doses have ranged up to 1 gram/day and CSF increases in both BH_4 and dopamine metabolites have been observed, but in most patients minimal effects on symptoms have been reported.[1,142-144,147,148]

CSF BH_4 is depleted over 50% in some dystonic patients.[1,143-144,149-152] However, unlike Parkinson's disease, the pathology of BH_4 depletion is unknown.[143] Nonetheless, consistent subjective and objective responses have been seen with BH_4 doses of 5–37 mg/kg as i.v. bolus or infusion,[1,143,151,152] and 5-hydroxytryptophan has been reported to enhance the effect of BH_4.[151] However, a close association between either serotonin or dopamine metabolites and dystonia, or remediation of symptoms, is not observed consistently.[152]

Biopterin levels in CSF and certain brain regions of patients with Alzheimer's disease are approximately half those of normal individuals, whereas other regions are unchanged.[1,142-144,153,154] To our knowledge, treatment of this disease with pterins has not been reported. Poor cellular transport of the aromatic

amino acids has been associated with infantile autisim.[155] Based upon this observation, BH_4 therapy for the disease has been attempted; successful treatment was reported in a double-blind trial.[156] Some patients suffering from depression have decreased CSF BH_4 levels.[1,157] Efficacy of BH_4 in subpopulations of depressed patients has been reported, but placebo effects and small patient populations in these studies make the results open to interpretation.[157-160] In contrast, plasma levels of biopterin in depressed individuals have been reported to be elevated 50–100% above control values.[1,161,162]

Hyperphenylalaninemia due to BH_4 deficiency. Atypical phenylketonuria (atypical PKU), hyperphenylalaninemia due to BH_4 deficiency, results from a deficiency in the phenylalanine hydroxylase cofactor, BH_4.[1,163-166] Since BH_4 also is the tyrosine hydroxylase and tryptophan hydroxylase cofactor as well, in most cases atypical PKU symptoms include severe neurological deficits.[1,163-166] Dhondt maintains an international registry of atypical PKU reports.[154] The incidence of the metabolic disease (or more properly, diseases) is approximately 5% of the total PKU population.[166] The disease is manifest in two main categories: those patients lacking BH_4 synthesis and those lacking DHPR, the BH_4 recycling enzyme. A subclass of the BH_4 biosynthesis deficiency has been found in which the patient demonstrates hyperphenylalaninemia, but does not present with the typical neurological defects of atypical PKU.[166,167] This subclass has been referred to as "peripheral" BH_4 deficiency because there appears to be adequate BH_4 for neurotransmitter synthesis, but not for normal phenylalanine hydroxylation in liver.[167] Prenatal diagnosis is available for all types of atypical PKU.[68]

Deficiencies in biosynthesis are characterized by decreases in either GTP-CH or PTP synthase,[167,169-172] with the latter being the most common form of the atypical disease.[166] Both deficiencies display low urinary biopterin levels; PTP synthase deficiency presents with abnormally high neopterin, while GTP-CH deficiency presents with low or no neopterin.[1,163-166] The peripheral form of BH_4 biosynthesis deficiency has been reported to be a result of a partial PTP synthase deficit.[167] Heterozygotes for both GTP-CH and PTP synthase deficiency have been detected.[167,170-172] Atypical PKU due to SR deficiency has not been reported. However, unclassified cases of BH_4 deficiency have been reported.[163-166]

Two animal models of BH_4 biosynthesis deficiency have been reported.[36,38] The GTP-CH inhibitor 2,4-diamino-6-hydroxypyrimidine has been shown to produce a functional peripheral BH_4 deficiency,[36] and the metabolic defect of the hph-1 mutant mouse is due to a decrease in GTP-CH.[38]

Deficiencies in DHPR activity result in inefficient recycling of quinonoid BH_2 to 7,8-BH_2, leading to an accumulation of oxidized forms of biopterin.[1,163-166,173] Since changes in biopterin and neopterin in this disease are not reliably large, assay of urinary pterin levels for detection of the disease is inadequate. Similarly, a BH_4 loading test to decrease serum phenylalanine cannot reliably discriminate between this disease and classical PKU. Rather, direct enzyme assay for Guthrie PKU test cards is recommended.[163-166,167] DHPR deficiency results in an apparent tetrahydrofolate deficiency in addition to the deficiency in BH_4.[174,175] Evidence has been presented to indicate that DHPR is responsible for maintaining both pterins and folates in the brain in the reduced state; thus, a DHPR deficiency would be expected to result in a loss of reduced folates.[163] The deficiency is exacerbated by folic acid treatment but is ameliorated by 5-formyltetrahydrofolate, which can replace the reduced folates directly.[174,175]

DHPR deficiency is genetically heterogeneous and results from at least two types of DHPR mutations.[176] An antibody to the normal enzyme has been used to demonstrate the complete absence of cross-reactive material in some patients and the presence of catalytically inactive DHPR in others.[176] In one case, the specific mutation was characterized as the insertion of an extra threonine codon.[177]

Chemotherapy for acute lymphoblastic leukemia can induce signs reminiscent of DHPR deficiency, that is, hyperphenylalaninemia, decreased levels of CSF-5-hydroxyindolacetic acid, and a higher than normal biopterin to neopterin ratio. BH_4 administration can reverse the hyperphenylalaninemia in these patients.[178]

Cofactor replacement therapy. While BH_4 does not cross the blood brain barrier well,[1,59,142-144,163-166,173] BH_4 replacement therapy for atypical PKU due to a defect in de novo BH_4 biosynthesis can be effective if treatment is started early.[163-166,173] In contrast, DHPR deficiency does not appear to be amenable to BH_4 replacement therapy,[163-166,173] presumably because there is a stoichiometric requirement for the cofactor in the absence of the recycling enzyme.[163]

The expense and poor brain penetration of BH_4 have led to the investigation of other pterins as replacement therapy for atypical PKU.[163,165,179] In one patient, 6-methyl-H_4pterin proved effective for nine months, but liver toxicities developed forcing a halt in the therapy.[163] In other patients, 6-methyl-H_4pterin was not successful.[163,165]

Synthetic programs and more extensive screening for new replacement cofactors have led to the development of new cofactor analogs for both phenylalanine hydroxylase and tyrosine hydroxylase with improved brain penetration.[180-184] However, none of these compounds has been tested clinically in either PKU or neurological diseases.

Abbreviations

BH_4 tetrahydrobiopterin
BH_2 dihydrobiopterin
NTP dihydroneopterin triphosphate
H_4pterin tetrahydropterin

H_2pterin dihydropterin
PTP pyruvoyl tetrahydropterin
DHPR dihydropteridine reductase
GTP-CH GTP cyclohydrolase
SR sepiapterin reductase
NAS N-acetyl serotonin
pbmc peripheral blood mononuclear cells
IFN interferon

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