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Mini Review

Maintenance of cellular tetrahydrobiopterin homeostasis

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Tetrahydrobiopterin (BH4) is a multifunctional cofactor of aromatic amino acid hydroxylases and nitric oxide synthase (NOS) as well as an intracellular antioxidant in animals. Through regulation of NOS activity BH4 plays a pivotal role not only in a variety of normal cellular functions but also in the pathogenesis of cardiovascular and neurodegenerative diseases, which develop under oxidative stress conditions. It appears that a balanced interplay between BH4 and NOS is crucial for cellular fate. If cellular BH4 homeostasis maintained by BH4 synthesis and regeneration fails to cope with increased oxidative stress, NOS is uncoupled to generate superoxide rather than NO and, in turn, exacerbates impaired BH4 homeostasis, thereby leading to cell death. The fundamental biochemical events involved in the BH4-NOS interplay are essentially the same, as revealed in mammalian endothelial, cardiac, and neuronal cells. This review summarizes information on the cellular BH4 homeostasis in mammals, focusing on its regulation under normal and oxidative stress conditions. [BMB reports 2010; 43(9): 584-592]

INTRODUCTION

6*R*-L-5,6,7,8-tetrahydrobiopterin (BH4) is widespread in eukaryotes, other than plants, and also occurs in cyanobacteria as various glycosides (1-3). In mammals, BH4 is not only an intracellular antioxidant to scavenge reactive oxygen species (ROS) but also a cofactor for aromatic amino acid hydroxylases (AAAHs), nitric oxide synthase (NOS, EC 1.14.13.39), and alkyl-glycerol monooxygenase (EC 1.14.16.5) (1). Phenylalanine hydroxylase is important for converting phenylalanine to tyrosine, particularly in the liver. Tyrosine and tryptophan hydroxylases are crucial for the synthesis of dopamine and serotonin, respectively, in the brain. All three forms of NOS (nNOS, iNOS, and eNOS) require BH4 as a key modulator to produce NO, which exhibits diverse vital functions throughout the human body.

BH4 deficiency is associated with a variety of diseases. Patients with congenital BH4 deficiency show hyperphenylalaninemia with severe neurological disorders and the DOPA-responsive form of dystonia, respectively (4). Recently, a genetic polymorphism in BH4 synthesis was found to determine susceptibility to pain (5). BH4 deficiency has also been studied extensively for possible associations with depression, autism, schizophrenia, Parkinson's disease, and Alzheimer's disease (1, 6-8). In addition, BH4 appears to play a fundamental role in the pathogenesis of vascular dysfunction resulting from oxidative stress conditions (9, 10).

BH4 distribution is highly tissue-specific in mammals, suggesting the presence of a tissue-specific regulatory mechanism to maintain cellular BH4 homeostasis (11, 12). Although BH4 is the predominant form of biopterins in normal plasma or cells, it is prone to oxidation to 7,8-dihydrobiopterin (BH2) by intracellular ROS. Inactive BH2 is reduced back to BH4 by regeneration enzymes to replenish cellular BH4 levels, which are also dependent on *de novo* synthesis from guanosine-5'-triphosphate (GTP). Because BH4/BH2 ratio as well as BH4 level determines the fate of NOS, they are under stringent control. Disturbed cellular BH4 homeostasis contributes to cellular dysfunction via regulation of NOS not only in vascular (9, 10, 13) but also in cardiac and brain systems (14-16).

Biosynthesis of BH4

BH4 is synthesized from GTP and regenerated from either quinonoid-dihydrobiopterin (q-BH2, 6,7-dihydrobiopterin) or BH2. Additionally, there is an alternative BH4 synthetic pathway catalyzed by the aldo-keto reductase family proteins and a salvage pathway beginning with sepiapterin (Fig. 1).

De novo synthesis

De novo synthesis of BH4 occurs by three enzymes; GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.2.3.12), and sepiapterin reductase (SR; EC 1.1.1.153) (1). The enzymatic product of GTPCH, dihydroneopterin triphosphate (H2-NTP), is transformed to 6-pyruvoyltetrahydropterin (PPH4) by PTPS and then further to BH4 by SR. SR performs two consecutive reductions of the C1′ carbonyl group via an isomerization reaction (17). Although SR alone completes the final step, aldose reductase (AR; 1.1.1.21) and carbonyl reductase (CR), which belong to the al-

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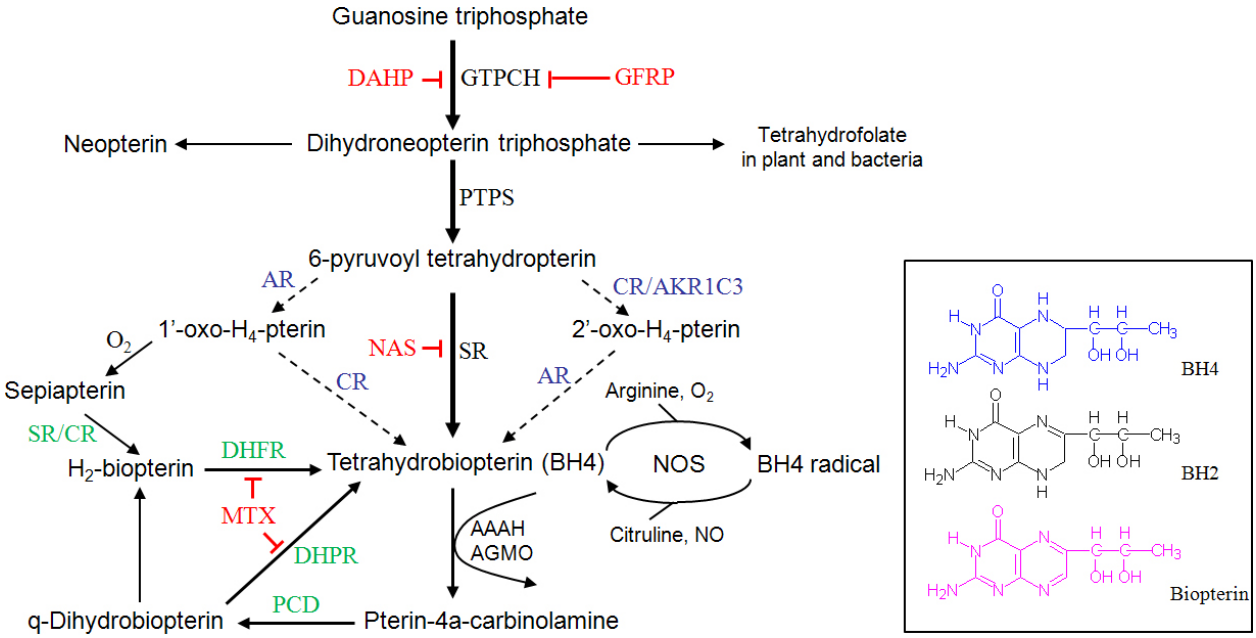

Fig. 1. *De novo* synthesis and regeneration of BH4. GTPCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyltetrahydropterin synthase; AR, aldose reductase; CR, carbonyl reductase; SR, sepiapterin reductase; DHFR, dihydrofolate reductase; DHPR, dihydropteridine reductase; GFRP, GTPCH feedback regulatory protein; AAAH, aromatic amino acid hydroxylases; AGMO, alkyl-glycerol monooxygenase; NOS, nitric oxide synthase; DAHP, 2,4-diamino-6-hydroxypyrimidine; NAS, N-acetyl serotonin; MTX, methotrexate.

do-keto reductase family, involve in this step (18, 19), constituting an alternative BH4 synthetic pathway. Recently, another aldo-keto reductase enzyme, 3α-hydroxysteroid dehydrogenase type 2 (AKR1C3, EC 1.1.1.213), was suggested to replace CR in the alternative pathway (20). Lastly, a salvage pathway begins with sepiapterin. Sepiapterin is converted to BH2 by SR and then to BH4 by dihydrofolate reductase (DHFR, EC 1.5.1.3). Although sepiapterin is not a normal endogenous substrate in animals, it can be produced nonenzymatically in vivo from 1'-oxo-tetrahydropterin, which is a product of AR from PPH4 in the absence of SR. DHFR mainly catalyzes the regeneration of tetrahydrofolate from dihydrofolate as a well-known target for the anticancer drug methotrexate (MTX). Both the alternative and salvage pathways contribute to BH4 synthesis *in vivo* when SR is insufficient, as demonstrated in patients deficient in SR (21, 22) and knockout mice disrupted in the SR gene (23).

Regeneration

BH4 is regenerated in two reactions. BH4 is oxidized to BH4-4a-carbinolamine during catalysis by AAAHs. BH4-4a-carbinolamine is converted to q-BH2 by pterin-4a-carbinolamine dehydratase (PCD/DCoH, EC 4.2.1.96) and then recycled back to BH4 by dihydropteridine reductase (DHPR, EC 1.6.99.7), which is also inhibited by MTX (24). PCD is a bifunctional protein, functioning as a dimerization cofactor of HNF-1 (DCoH) in the nucleus. Because there is an isozyme, DCoHα, which shares 60% identity with DCoH and has similar catalytic efficiency (25), hyperphenylalaninemia is transient in patients with a PCD deficiency. In the absence of DHPR, q-BH2, which is quite unstable, is nonenzymatically rearranged to the stable BH2 isomer.

Regulation of *de novo* BH4 synthesis

While *de novo* synthesis increases the pool of total biopterin, the regeneration pathway is crucial for maintaining a high BH4/BH2 ratio. *De novo* synthesis of BH4 is thought to be tightly regulated, because the intracellular levels of BH4 are at or below the saturating levels for the enzymes utilizing BH4 (26). Primary regulation is given to GTPCH, as the committing and rate-limiting enzyme of BH4 synthesis (1).

Allosteric regulation of GTPCH

GTPCH activity is tightly regulated by cellular levels of phenylalanine and BH4 via a GTPCH feedback regulatory protein (GFRP) (Fig. 2), which was first isolated from rat liver (26). Mammalian GTPCH is a homodecameric protein sandwiched by two homopentameric GFRPs in the presence of BH4 and GTP or phenylalanine alone (27, 28). GFRP mediates allosteric control of GTPCH by the ligands (Fig. 2): GFRP induces feedforward activation of GTPCH in the presence of phenyl-

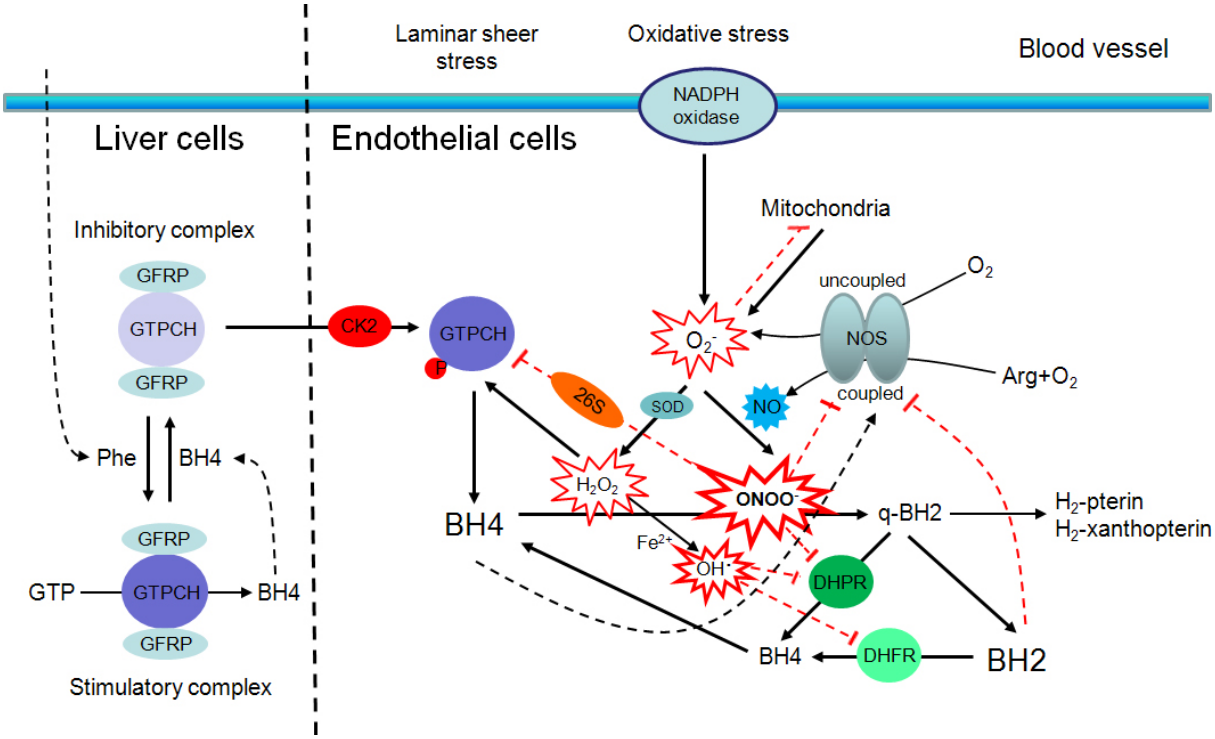

Fig. 2. Schematic diagram depicting the regulation of GTPCH activity and BH4 homeostasis. (left) GFRP mediated regulation of GTPCH in the liver. (right) Regulation of BH4 homeostasis in endothelial cells. GTPCH phosphorylation induced by laminar shear stress under normal conditions and the biochemical events leading to the impairment of BH4 and NO homeostases under oxidative stress conditions. BH4, tetrahydrobiopterin; BH2, 7,8-dihydrobiopterin; q-BH2, quinonoid dihydrobiopterin; GTPCH, GTP cyclohydrolase I; DHFR, dihydrofolate reductase; DHPR, dihydropteridine reductase; GFRP, GTPCH feedback regulatory protein; NO, nitric oxide; NOS, nitric oxide synthase; CK2, casein kinase 2; 26S, 26S proteasome; SOD, superoxide dismutase; Arg, arginine; Phe, phenylalanine.

alanine, whereas it induces feedback inhibition of GTPCH in the presence of BH4. In the absence of GFRP, GTPCH displays strong positive cooperativity with the GTP substrate. GFRP seems to parallel GTPCH in the expression levels, as they are highly expressed in liver and kidney and lower in brain and other tissues (29, 30). Thus, GFRP acts as a fundamental supervisor to control GTPCH activity in the presence of subsaturating concentrations of intracellular GTP (28).

Post-translational regulation of GTPCH

GTPCH activity is regulated by phosphorylation (Fig. 2). Recently, phosphorylation of GTPCH in human aortic endothelial cells was shown to be regulated by laminar shear stress (31), which agrees well with the physiological milieu of endothelial cells. GTPCH is phosphorylated on Ser81 by CK2, which is a protein serine/threonine kinase ubiquitous in eukaryotes (32), when stimulated by unidirectional laminar shear stress but not by bidirectional oscillatory shear stress. The GTPCH/GFRP complex is dissociated by laminar shear, and the free GTPCH is subject to phosphorylation, producing more BH4 and thus more NO, leading to vasorelaxation (31, 33). Phosphorylation of GTPCH was also studied previously in PC12 cells (34), rat kidney mesangial cells (35), and mouse bone marrow-derived mast cells (36). Interestingly, GTPCH is phosphorylated transiently in Ag/IgE-induced mast cells, accompanied by increased NO production (37). GTPCH phosphorylation is thought to be common in other cell types through signals that are unique to each of them. According to a systematic study that examined potential phosphorylation sites in rat GTPCH, phosphorylation occurs at multiple serine or threonine sites (38), in addition to S72 that corresponds to S81in the human sequence. Furthermore, mutation of the phosphorylation sites affects nuclear localization of GTPCH, which is already known in COS-1 cells (39), epidermal keratinocytes, and melanocytes (40), although the functional meaning remains unknown.

New findings have suggested additional potential regulation of GTPCH activity. GTPCH appears to exist as a multiprotein complex with cross-talk to other proteins *in vivo*. GTPCH is degraded by proteasome 26S in human umbilical vein endothelial cells in high glucose (41, 42) and in aortas isolated from streptozotocin-injected diabetic mice (42). A proteomic study in adult human astrocytes showed that various adaptor/regulator proteins including CK2α and JNK3 are associated with

GTPCH, and their levels changed after treatment with a cytokine trio (interferon-γ, tumor necrosis factor-α, and IL-1β), which stimulated GTPCH activity up to 40-fold (43). Notably, GTPCH is found in caveolae of mouse vascular endothelial cells, along with caveolin-1 and eNOS (44), which are colocalized in the microdomain (45). Caveolae occupy up to 30% of the cell surface in capillaries and harbor many different proteins.

Transcriptional regulation of GTPCH

Inflammatory mediators, such as lipopolysaccharide, increase GTPCH transcription via the cytokine trio (IFN-γ, TNF-α, and IL-1β) in various cell types including endothelial cells and human astrocytes. Furthermore, GTPCH expression is regulated by various factors (46, 47), and more factors are being added to the list together with signaling mediators. It is no wonder that the GFRP mRNA level is downregulated by the cytokine trio; thus, decreasing the GTPCH/GFRP complex and enhancing BH4 synthesis (48, 49). However, GFRP mRNA expression is upregulated in smooth muscle cells of rat aorta (50) and adult rat cardiac myocytes (51). Moreover, GTPCH activity is not affected by changes in GFRP expression, suggesting that endothelial GTPCH expression is the primary determinant of BH4 levels (52). In human umbilical vein endothelial cells, phenylalanine enhances GTPCH mRNA expression but not that of GFRP (30). These discrepancies may reflect the presence of a tissue-specific mechanism to regulate cellular BH4 homeostasis.

Regulation of PTPS and SR

Sporadic reports exist on the mild upregulation of PTPS and SR expression by cytokines or other factors (53, 54). When GTPCH expression is stimulated in human and primate phagocytes, where PTPS activity is low and is not upregulated (55), the intermediate H2-NTP accumulates and is converted to neopterin, which is detected in plasma as an indirect marker of inflammation (1). Kinetic and X-ray crystallography studies of rodent brain SR suggest a highly specific feedback regulatory mechanism of SR by endogenous *N*-acetyl-serotonin, which has a K_i value of 0.2 μM (56, 17). Additionally, the presence of an alternative pathway catalyzed by aldo-keto reductases may provide a tissue-specific heterogeneity in BH4 homeostasis, as shown in SR patients presenting with neurotransmitter deficiencies in the brain without hyperphenylalaninemia (21).

BH4 regeneration and NOS function

As demonstrated in DHPR patients, who accumulate BH2 in their body fluids and develop hyperphenylalaninemia with severe brain abnormalities (1, 57), successful regeneration of BH4 is necessary for proper functioning of AAAHs. BH4 regeneration is also important for NOS function. As a prosthetic group in NOS, BH4 acts as a single-electron donor during arginine hydroxylation and is then reduced back to BH4 via a radical intermediate within NOS before further catalysis can proceed (58, 59). Thus, continuous BH4 regeneration does not appear to be required for normal NOS activity *in vivo*. However, a high BH4/BH2 ratio is indispensable to NOS function.

NOS uncoupling

Intracellular ROS diminishes BH4/BH2 ratio through accelerated oxidation of BH4 to BH2 (60, 61). Although NO is released from NOS under normal conditions, insufficient availability of BH4 to NOS results in the formation of superoxide rather than NO (termed NOS uncoupling) (62, 63). Furthermore, as BH4 and BH2 bind eNOS with equal affinity, the increased BH2 rapidly replaces NOS-bound BH4 (64), further deteriorating NOS uncoupling (65). NOS uncoupling is also common in nNOS and iNOS (66, 67).

Interplay between BH4 and NOS

The intracellular biochemical events leading to disruption of BH4 homeostasis have been well studied in endothelial cells (Fig. 2). NADPH oxidase, the mitochondrial electron transport chain, and uncoupled NOS are the three major ROS sources within the cell, and they have significant interactions. At the initial stage of oxidative stress, NADPH oxidase is the main superoxide generator (68, 69). The superoxide reacts rapidly with NO to produce peroxynitrite (70), eliminating the NO for vasodilation. Peroxynitrite, which is a strong biological oxidant, is more potent than either superoxide or H_2O_2 to oxidize BH4 to either BH2 or dihydropterin and dihydroxanthopterin via q-BH2 (60, 71, 72). Furthermore, a hydroxyl radical generated from H_2O_2 by the Fenton reaction may also participate in BH4 destruction (73). Although not verified *in vivo* in association with BH4, the toxicity of Fe^{2+} and Cu^{2+} is quite well known in diseases of aging, including atherosclerosis (74). The unstable intermediate q-BH2 is converted nonenzymatically to BH2 and then catalyzed by DHFR to BH4. Otherwise, q-BH2 might be reduced back to BH4 by DHPR. If DHPR and/or DHFR activity override the rate of BH4 oxidation, the BH4/BH2 ratio recovers to a high level and NOS remains in a coupled state. The stable ROS H_2O_2 produced from superoxide may upregulate BH4 synthesis, as shown in mouse brain microvascular endothelial cells (75, 76). If BH4 is not augmented and the BH4/BH2 ratio is diminished, eNOS becomes uncoupled and generates superoxide rather than NO, deteriorating the situation. Both DHPR and DHFR are presumed be inactivated and/or downregulated under conditions of persistent superoxide generation. Furthermore, an *in vitro* study with human eNOS showed that peroxynitrite oxidizes BH4 bound to the enzyme and then disrupts the heme center of the enzyme, irreversibly destabilizing the dimer (77). Under chronic oxidative conditions, uncoupled eNOS becomes a feed-forward trigger. NOS is said to be a Janus-faced enzyme (78). Coupled eNOS produces NO, whereas uncoupled eNOS generates superoxide. An essential determinant of the choice is BH4/BH2 ratio as well as BH4 level (64) but the reverse is also true. In short, a balanced interplay between BH4 and NOS

maintains cellular function.

The role of DHFR in regulating BH4 homeostasis was shown first in bovine aortic endothelial cells, where DHFR expression is downregulated by H_2O_2 application and angiotensin II stimulation, accompanied by reduced BH4 levels (79). Subsequently, a genetic knockdown study in murine endothelial cells showed that BH4/BH2 ratio was diminished by DHPR knockdown but not by GTPCH knockdown (80), thereby supporting the crucial role of DHFR in maintaining BH4/BH2 ratio, which was suggested to be the primary determinant of eNOS coupling rather than the absolute levels of BH4 (64). On the other hand, there is no direct evidence if DHPR collaborates with DHFR for BH4 regeneration in endothelial cells. Nevertheless, several sporadic reports suggest a possible association of DHPR. An interesting finding was that the DHPR protein decreases progressively in aortic smooth muscles of spontaneously hypertensive rats (81). DHPR activities in the white-blood cells are low in patients with vitiligo compared to normal subjects (82). DHPR activities in erythrocytes from patients with insulin-resistant diabetes are lower than normal subjects (83). Human brain DHPR is irreversibly inactivated *in vitro* when incubated with L-dopa (84) or H_2O_2 (82). Maintenance of BH4 regeneration in endothelial cells may be accomplished by combined actions of both DHPR and DHFR or either one of them.

The biochemical events leading to NOS uncoupling appear to occur commonly in systemic and pulmonary vascular disorders (68, 85, 86), heart failure (14), and in neuronal cell death (15, 87, 88). GTPCH-deficient mice, which constitutively express reduced levels of GTPCH protein, have pulmonary hypertension but not systemic hypertension due to abnormal BH4-dependent eNOS regulation (85, 86). Diastolic dysfunction in a heart-failure hypertensive mouse model is due to NOS uncoupling resulting from a diminished BH4/BH2 ratio in cardiac myocytes (14). Although both eNOS and nNOS exist in the heart (89), the selective nNOS inhibitor inhibits cardiac superoxide production more than an iNOS inhibitor (14). However, DHFR mRNA and protein are not detected in adult rat cardiac myocytes (51). According to MPP^+-induced toxicity studies in rat cerebellar granule neurons (15) and neuroblastoma cells transfected with human nNOS (87), MPP^+ increases superoxide generation, probably through impaired mitochondria, and diminishes the BH4/BH2 ratio. A timely report demonstrated the demise of SH-SY5Y neuroblastoma cells and mouse primary cortical neurons due to disturbed cellular BH4 homeostasis (88).

CONCLUSIONS

From the foregoin g discussion, it is apparent that a core event of cellular BH4 homeostasis under oxidative stress conditions is the interplay between BH4 and NOS. A simplified scheme emphasizing the balanced interplay between them is presented in Fig. 3. Both BH4 level and BH4/BH2 ratio must be maintained for cellular BH4 homeostasis, which is independent from plasma BH4 homeostasis. Under conditions of increased oxidative stress, BH4 plays a protective role for NOS against ROS, being oxidized to BH2. DHFR and/or DHPR recycle the oxidized BH2 to BH4 to replenish the intracellular BH4 pool, while upregulated BH4 synthesis via stimulation of GTPCH expression/activity increases the pool. If BH4 is insufficient, NOS becomes uncoupled and generates superoxide. The increased ROS further aggravates the oxidative situation. This feed-forward interplay between the diminished BH4/BH2 ratio and NOS uncoupling constitutes a fundamental event in cellular pathogenesis. Therefore, genetic and/or environmental factors that disturb cellular BH4 homeostasis may exert detrimental effects on the system.

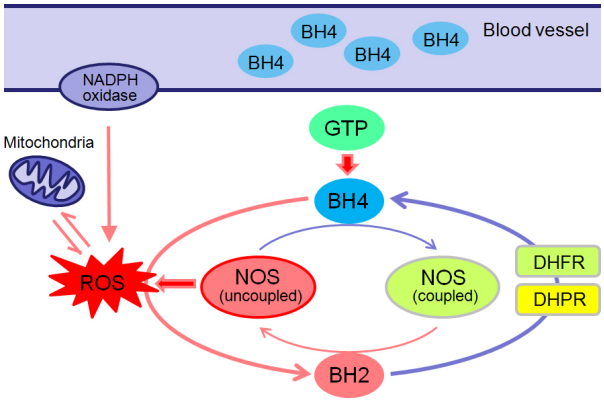

Fig. 3. A simplified scheme of the interplay between BH4 and nitric oxide synthase (NOS) to maintain BH4 cellular homeostasis. GTP, guanosine 5′-triphosphate; DHFR, dihydrofolate reductase; DHPR, dihydropteridine reductase; BH4, tetrahydrobiopterin; BH2, 7,8-dihydrobiopterin; ROS, reactive oxygen species.

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