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Tetrahydrobiopterin Supplementation Improves Phenylalanine Metabolism in a Murine Model of Severe Malaria

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Abstract

Tetrahydrobiopterin (BH₄) is an essential cofactor for both phenylalanine hydroxylase and nitric oxide synthase. Patients with severe malaria have low urinary BH₄, elevated plasma phenylalanine, and impaired endothelium-dependent vasodilation, suggesting that BH₄ depletion may limit phenylalanine metabolism and nitric oxide synthesis. We infected C57BL/6 mice with *Plasmodium berghei* ANKA to characterize BH₄ availability and to investigate the effects of BH₄ supplementation. *P. berghei* ANKA infection lowered BH₄ in plasma, erythrocytes, and brain tissue but raised it in aorta and liver tissue. The ratio of BH₄ to 7,8-BH₂ (the major product of BH₄ oxidation) was decreased in plasma, erythrocytes, and brain tissue, suggesting that oxidation contributes to BH₄ depletion. The continuous infusion of sepiapterin (a BH₄ precursor) and citrulline (an arginine precursor) raised the concentrations of BH₄ and arginine in both blood and tissue compartments. The restoration of systemic BH₄ and arginine availability in infected mice

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Supporting Information

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Effects of sepiapterin/citrulline on dietary intake and whole blood nitrite (PDF)

The authors declare no competing financial interest

produced only a minor improvement in whole blood nitrite concentrations, a biomarker of NO synthesis, and failed to prevent the onset of severe disease symptoms. However, sepiapterin and citrulline infusion reduced the ratio of phenylalanine to tyrosine in plasma, aortic tissue, and brain tissue. In summary, BH4 depletion in *P. berghei* infection may compromise both nitric oxide synthesis and phenylalanine metabolism; however, these findings require further investigation in human patients with severe malaria.

Keywords

metabolism; host response; plasmodium; nitric oxide; endothelium

(6*R*)-5,6,7,8-Tetrahydrobiopterin (BH4) is an essential cofactor for phenylalanine hydroxylase (PAH) and nitric oxide synthase (NOS).^{1,2} Urinary BH4 is low in both pediatric³ and adult⁴ severe malaria. Severe malaria is characterized by hyperphenylalaninemia,^{5–7} impaired vasodilation,⁸ and low plasma and urinary nitrite and nitrate concentrations.⁹ We hypothesized that low BH4 may contribute to severe malaria pathogenesis by impairing PAH and NOS activity.

BH4 synthesis and recycling are dependent on reducing equivalents provided by NADH and NADPH, thus BH4 bioavailability is reflective of the cellular redox status. BH4 is synthesized from guanosine-5'-triphosphate (GTP) by GTP cyclohydrolase (GTPCH), 6-pyruvoyl tetrahydrobiopterin synthase (PTPS), and sepiapterin reductase (SR), with the final step requiring 2 NADPH reducing equivalents^{10,11} (Figure 1). In mammalian cells, the GTPCH-regulated conversion of GTP to 7,8-dihydroneopterin triphosphate is the rate-limiting step in this pathway.¹² Under physiological conditions, BH4 can react with molecular oxygen,¹³ peroxynitrite,¹⁴ or other free radical species¹⁵ to produce 7,8-BH2. Dihydrofolate reductase (DHFR) utilizes 1 reducing equivalent of NADPH to reduce 7,8-BH2 to BH4.^{16,17}

The conversion of phenylalanine to tyrosine by PAH was the first enzymatic reaction discovered to be BH4-dependent.¹⁸ BH4 facilitates the binding of oxygen to the nonheme iron at the PAH active site. In the process of producing a highly reactive Fe(IV) oxo complex ultimately to hydroxylate phenylalanine, BH4 is oxidized to tetrahydrobiopterin-4a-carbinolamine (or 4a-hydroxy-BH4).¹¹ BH4 is thus consumed in equal stoichiometry to tyrosine production. Catalytically active BH4 is restored by the sequential actions of pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) via the intermediate quinonoid-BH2 (q-BH2). The second step in this recycling pathway requires 1 reducing equivalent of NADH to convert quinonoid-BH2 to fully reduced BH4 (Figure 1).

Nitric oxide synthesis is also dependent on BH4 bioavailability. As a NOS cofactor, BH4 transiently donates an electron to produce a BH4^{•+} radical, which facilitates arginine oxidation and formation of the Fe²⁺-NO complex.¹⁹ However, BH4^{•+} recaptures an electron during the release of NO from the heme catalytic center, thereby restoring fully reduced BH4.^{20,21} Under conditions of low BH4 availability, arginine oxidation is uncoupled from NO synthesis and NOS generates superoxide (O₂^{•−}) instead.^{22–26} The major BH4 oxidation product 7,8-BH2 is catalytically inactive and can compete with BH4 for binding to NOS,

thereby promoting NOS uncoupling.^{25,26} In both pediatric and adult patients with severe malaria, urinary 7,8-BH2 was found to be increased,^{3,4} suggesting that oxidation could contribute to BH4 depletion and that 7,8-BH2 accumulation could contribute to NOS uncoupling. A recent intravital microscopy study demonstrated impaired vasodilation of pial vessels in *P. berghei* ANKA-infected mice following acetylcholine or NMDA stimulation.²⁷ Superfusion of BH4 improved acetylcholine- and NMDA-induced pial vasodilation in infected mice.²⁷ This finding implies that BH4 depletion limits NO synthesis in the cerebral vasculature during malaria infection. However, the bioavailability of BH4 and 7,8-BH2 in blood or tissues has never been directly measured in the setting of severe malaria.

RESULTS

Tetrahydrobiopterin Bioavailability in Blood and Tissues of *P. berghei* ANKA-Infected Mice.

We used HPLC to quantify BH4 in plasma and erythrocyte samples collected from *P. berghei* ANKA-infected mice on day 6 postinoculation; uninfected littermates served as controls. Both plasma and erythrocyte BH4 were substantially lower in infected mice compared to uninfected controls (plasma, 0.09 [0.07–0.16] vs 0.23 [0.22–0.27] $\mu\text{mol/L}$, $p < 0.0001$; erythrocytes, 0.22 [0.20–0.27] vs 0.77 [0.73–0.78] $\mu\text{mol/L}$, $p < 0.0001$, Figure 2). The BH4 oxidation product 7,8-BH2 was also lower in erythrocytes of infected mice compared to uninfected controls (0.09 [0.08–0.11] vs 0.24 [0.22–0.26] $\mu\text{mol/L}$, $p < 0.0001$) but did not differ in plasma (Figure 2). The BH4/7,8-BH2 ratio was lower in both plasma and erythrocytes from infected mice compared to uninfected controls (plasma, 1.2 [1.2–1.8] vs 3.0 [2.7–3.0], $p < 0.0001$; erythrocytes, 2.7 [2.1–3.0] vs 3.2 [3.0–3.5], $p < 0.001$, Figure 2). These findings imply that *P. berghei* ANKA infection increases the oxidation of BH4 to 7,8-BH2 in the blood. Total biopterin concentrations (BH4 + 7,8-BH2) were also decreased in both plasma and erythrocytes, suggesting that de novo BH4 synthesis in blood or transport from tissues is impaired in during *P. berghei* ANKA infection.

In brain tissue, *P. berghei* ANKA infection lowered the concentration of BH4 (14.8 [13.1–16.5] vs 18.1 [17.0–20.8] pmol/mg protein, $p < 0.001$) and raised the concentration of 7,8-BH2 (2.1 [1.7–2.2] vs 1.0 [0.8–1.3] pmol/mg protein, $p < 0.0001$), lowering the BH4/7,8-BH2 ratio substantially (7.6 [6.4–8.0] vs 17.4 [14.3–22.0], $p < 0.0001$, Figure 2). In aortic tissue, *P. berghei* ANKA infection raised the concentration of BH4 (49.0 [33.7–52.8] vs 32.5 [24.9–41.4] pmol/mg protein, $p < 0.05$) but did not significantly increase the 7,8-BH2 concentration ($p = 0.07$) or the BH4/7,8-BH2 ratio ($p = 0.55$, Figure 2). In liver tissue, *P. berghei* ANKA infection raised the BH4 concentration (80.1 [72.3–89.9] vs 60.2 [57.8–69.8] pmol/mg protein, $p < 0.05$), raised the 7,8-BH2 concentration (6.2 [5.3–6.6] vs 4.8 [3.7–5.5] pmol/mg protein, $p < 0.01$), but did not raise the BH4/7,8-BH2 ratio ($p = 0.54$, Figure 2).

Phenylalanine and Tyrosine Metabolism in *P. berghei*-Infected Mice.

Plasma phenylalanine was higher in *P. berghei* ANKA-infected mice compared to uninfected controls (112.6 [105.0–116.1] vs 70.4 [57.5–80.3] $\mu\text{mol/L}$, $p < 0.0001$, Figure 3) whereas plasma tyrosine was lower (58.1 [44.8–67.4] vs 77.4 [67.7–94.6] $\mu\text{mol/L}$, $p < 0.0001$). As a result, the phenylalanine/tyrosine ratio was elevated in infected mice (2.0 [1.8–2.4] vs 0.9 [0.8–1.0], $p < 0.0001$, Figure 3). *P. berghei* ANKA infection also elevated the phenylalanine/

tyrosine ratio in aorta (1.4 [1.3–1.6] vs 0.9 [0.9–1.0], $p < 0.001$), liver (1.4 [1.3–1.5] vs 1.1 [1.0–1.1], $p < 0.001$), and brain (1.8 [1.7–2.1] vs 0.9 [0.8–1.0], $p < 0.001$, Figure 3). These data imply that phenylalanine hydroxylase activity is impaired during malaria infection.

Arginine Bioavailability in Blood and Tissues of *P. berghei*-Infected Mice.

There was less plasma citrulline, arginine, and ornithine in infected mice than in uninfected littermates (Figure 4). In brain tissue, the citrulline concentration could not be determined, the arginine concentration was lower, and the ornithine concentration was unchanged in mice infected with *P. berghei* ANKA (Figure 4). In aortic tissue, the amount of citrulline was lower, whereas the amounts of arginine and ornithine were unchanged in mice infected with *P. berghei* ANKA (Figure 4). In liver tissue, the amount of citrulline was unchanged, whereas the amounts of both arginine and ornithine were elevated in mice infected with *P. berghei* ANKA (Figure 4).

Sepiapterin and Citrulline Infusion Raised the Tetrahydrobiopterin Bioavailability in Blood and Tissues and Lowered the Phenylalanine/Tyrosine Ratio.

To assess the impact of BH4 and arginine depletion on PAH and NOS activity, we coinfused sepiapterin (a BH4 precursor) and citrulline (an arginine precursor) into *P. berghei* ANKA-infected mice. Compared to the infusion of saline, the infusion of sepiapterin and citrulline raised the plasma concentrations of BH4 (0.76 [0.61–1.28] $\mu\text{mol/L}$, $p < 0.0001$) and 7,8-BH2 (0.58 [0.53–0.89] $\mu\text{mol/L}$, $p < 0.0001$) but did not raise the BH4/7,8-BH2 ratio (1.2 [0.8–1.6] $\mu\text{mol/L}$, $p = 0.15$, Figure 2). In erythrocytes, sepiapterin and citrulline infusion raised the BH4 (3.12 [2.75–6.45] $\mu\text{mol/L}$, $p < 0.0001$) and 7,8-BH2 (0.91 [0.77–1.63] $\mu\text{mol/L}$, $p < 0.0001$) levels and the BH4/7,8-BH2 ratio (3.9 [3.5–4.0], $p < 0.001$, Figure 2). Compared to the infusion of saline, the infusion of sepiapterin and citrulline into *P. berghei* ANKA-infected mice raised both the BH4 and 7,8-BH2 levels in brain, aortic, and liver tissues ($p < 0.0001$ for each comparison) but had no effect on the BH4/7,8-BH2 ratios (Figure 2).

To determine if BH4 supplementation improved phenylalanine metabolism, we assessed phenylalanine and tyrosine concentrations in infected mice infused with sepiapterin and citrulline. Sepiapterin and citrulline infusion decreased the plasma phenylalanine concentration from 112.6 [105.0–116.1] $\mu\text{mol/L}$ in saline-treated mice to 98.9 [83.3–109.0] $\mu\text{mol/L}$ in sepiapterin/citrulline-treated mice ($p < 0.05$), increased the plasma tyrosine concentration from 58.1 [44.8–67.4] to 68.0 [64.5–76.6] $\mu\text{mol/L}$ ($p < 0.05$), and decreased the phenylalanine/tyrosine ratio from 2.0 [1.8–2.4] to 1.3 [1.2–1.7] ($p < 0.0001$, Figure 3). Sepiapterin and citrulline infusion also reduced the phenylalanine/tyrosine ratio in the aorta from 1.4 [1.3–1.6] to 1.2 [1.1–1.4] ($p < 0.05$) and in the brain from 1.8 [1.7–2.1] to 1.4 [1.2–1.6] ($p < 0.001$, Figure 3). The phenylalanine/tyrosine ratio also decreased from 1.4 [1.3–1.5] to 1.3 [1.2–1.4] in the liver tissue of infected mice treated with sepiapterin and citrulline, but this difference did not reach statistical significance ($p = 0.09$, Figure 3). Taken together, these observations suggest that malaria-associated oxidation and depletion of tetrahydrobiopterin limits phenylalanine hydroxylase activity, leading to an elevation of plasma and tissue phenylalanine concentrations. The administration of sepiapterin and

citrulline in *P. berghei* ANKA-infected mice increases the tetrahydrobiopterin availability and improves the metabolism of phenylalanine to tyrosine.

Sepiapterin and Citrulline Infusion Raised the Arginine Bioavailability in Plasma and Tissues of *P. berghei*-Infected Mice and Increased Nitrite.

Compared to saline infusion, sepiapterin and citrulline infusion raised citrulline, arginine, and ornithine concentrations in the plasma of infected mice (Figure 4). Sepiapterin and citrulline infusion also raised the arginine and ornithine concentrations in the brain tissue of *P. berghei* ANKA-infected mice and raised the citrulline, arginine, and ornithine concentrations in aortic tissue of *P. berghei*-infected mice (Figure 4). In the liver tissue of infected mice, sepiapterin and citrulline infusion raised the concentration of citrulline and ornithine (Figure 4). Liver arginine, which was already elevated by infection, did not increase further with sepiapterin and citrulline infusion (Figure 4).

We hypothesized that sepiapterin and citrulline infusion would improve NO synthesis by restoring BH4 and arginine. Careful studies in human subjects have established nitrite as a superior biomarker of eNOS activity compared to nitrate,²⁸ and subsequent studies have confirmed the utility of nitrite as an eNOS biomarker in mice.²⁹ The nitrite concentration was lower in *P. berghei* ANKA-infected mice infused with saline (0.28 [0.25–0.34] $\mu\text{mol/L}$) than in uninfected mice infused with saline (0.33 [0.27–0.43] $\mu\text{mol/L}$), but this difference was not statistically significant ($p = 0.34$). The infusion of sepiapterin and citrulline raised the whole blood nitrite level in *P. berghei* ANKA-infected mice from 0.28 [0.25–0.34] to 0.35 [0.32–0.40] $\mu\text{mol/L}$ ($p < 0.05$ vs infected + saline, Figure 5).

In separate experiments that examined the effects of dietary intake on whole blood nitrite, we analyzed whole blood samples from uninfected mice that were provided with only as much food as was consumed by matched *P. berghei* ANKA-infected mice. Dietary restriction decreased whole blood nitrite to a level similar to *P. berghei* ANKA-infected mice (Supporting Information, Figure S1). Because sepiapterin/citrulline infusion improved the dietary intake of infected mice (Figure S2), we cannot exclude the possibility that elevated blood nitrite in the sepiapterin/citrulline-infused group was due to increased dietary intake rather than a specific effect of BH4 and arginine on NOS activity.

Effect of BH4 and Arginine Supplementation on Behavioral Symptoms of *P. berghei* Infection.

To determine the effect of BH4 and arginine cosupplementation on disease progression, we quantified behavioral symptoms in cohorts of treated and untreated *P. berghei* ANKA-infected mice. On day 6 postinoculation, *P. berghei* ANKA-infected mice infused with sepiapterin and citrulline had higher median behavioral scores than infected controls infused with saline on the morning of day 6 postinoculation (6 [0–13] vs 2 [0–7], $p = 0.37$ vs infected + saline) and directly before tissue collection (1.5 [1–12] vs 1 [0–3], $p = 0.10$ vs infected + saline), but these differences did not reach statistical significance (Figure 6).

DISCUSSION

This study is the first to identify BH4 depletion in the plasma, erythrocytes, and brain tissue of *P. berghei* ANKA-infected mice and to investigate the effects of systemic treatment with sepiapterin and citrulline. In plasma, erythrocytes, and brain tissue, we found decreased ratios of BH4 to 7,8-BH2, a product of BH4 oxidation, indicating that infection-associated oxidative stress or impaired recycling of 7,8-BH2 may contribute to BH4 depletion. Continuous coinfusion of sepiapterin and citrulline in *P. berghei* ANKA-infected mice raised plasma and tissue levels of BH4 and arginine and improved the BH4/7,8-BH2 ratio in erythrocytes. Despite meeting the pharmacological end point of raising BH4 and arginine availability, whole blood nitrite (a biomarker of NO synthesis) was only marginally improved and infused mice were not substantially protected from symptoms of severe disease. Our results agree with and extend the findings from a study in which *P. berghei* ANKA-infected mice treated with twice-daily intraperitoneal injection of arginine (~400 mg/kg/day) or BH4 (~100 mg/kg/day) had higher levels of blood nitrite than untreated infected mice.⁶¹ We extended this observation by directly measuring biopterins and amino acids in blood and tissue, by using the precursors sepiapterin and citrulline that are converted to arginine and BH4 intracellularly, and by examining the effects not only on nitric oxide synthesis but also on phenylalanine metabolism. The simultaneous infusion of sepiapterin and citrulline reduced the phenylalanine/tyrosine ratios in the plasma, brain, and aorta of *P. berghei* ANKA-infected mice. This finding suggests that the restoration of BH4 availability improved the activity of phenylalanine hydroxylase, which converts phenylalanine to tyrosine.

In patients with phenylketonuria, an inborn loss-of-function mutation of the phenylalanine hydroxylase gene causes hyperphenylalaninemia and severe neurological complications, including mental retardation, attention deficit disorder, and neuromotor impairment.^{30,31} Hyperphenylalaninemia and neurological disease also occur in individuals with loss-of-function mutations in the gene encoding GTP cyclohydrolase (GTPCH), which is required for de novo BH4 biosynthesis. Large phase III trials demonstrated that sapropterin, an orally available preparation of synthetic 6R-BH4, significantly reduced plasma phenylalanine levels in individuals with PKU with minimal side effects.^{34,35} Patients with severe malaria do not exhibit the extreme concentrations (>1000 $\mu\text{mol/L}$) observed in poorly controlled phenylketonuria, reducing the likelihood that direct phenylalanine toxicity contributes to severe malaria pathogenesis.^{32,33} However, impaired phenylalanine hydroxylation could interfere with the synthesis of dopamine, epinephrine, and norepinephrine.

Low BH4/7,8-BH2 ratios and decreased BH4/NOS stoichiometry have been shown to favor NOS uncoupling, resulting in decreased NO synthesis and increased production of the pro-oxidant superoxide.^{16,25} In the brains of *P. berghei* ANKA-infected mice, we observed decreased BH4 and elevated 7,8-BH2, resulting in decreased BH4/7,8-BH2 ratios. Immunohistochemical studies in brain tissue from children who died of severe malaria have demonstrated increased inducible nitric oxide synthase (iNOS) staining,^{37,38} and eNOS and iNOS protein levels are elevated in *P. berghei* ANKA-infected mice.²⁷ Increased NOS protein in the setting of BH4 depletion would exacerbate dysregulation of BH4/NOS stoichiometry and favor NOS uncoupling. In that study, *P. berghei* ANKA was also shown to

increase the fraction of inactive nNOS monomers in brain tissue of infected mice, although nNOS protein levels and the phosphorylation state were normal. Direct superfusion of BH4 improved pial vasodilation in response to acetylcholine or NMDA, suggesting that BH4 depletion affected both eNOS and nNOS function.²⁷ These findings also imply that BH4 depletion may impact nNOS function with regard to neurotransmission.

In our experiments, the infusion of sepiapterin and citrulline improved BH4 in brain tissue but failed to correct brain BH4/7,8-BH2 ratios. Similarly, a recent study of patients with coronary artery disease found that oral BH4 treatment increased plasma and saphenous vein tissue BH4 concentrations but concomitantly increased 7,8-BH2 concentrations, yielding no improvement in the BH4/7,8-BH2 ratio.³⁶ Failure to improve BH4/7,8-BH2 ratios may explain the lack of effect of treatment on brachial flow-mediated vasodilation, aortic or carotid distensibility, or acetylcholine-induced vasodilation in ex vivo saphenous vein rings.³⁶ Failure to correct the BH4/7,8-BH2 ratio may also induce limited restoration of NO synthesis in mice infused with sepiapterin and citrulline in our experiments. In our experiments, NO synthesis may also be limited by impaired eNOS phosphorylation, which has been observed in the brain tissue of *P. berghei* ANKA-infected mice,²⁷ or by accumulation of the NOS inhibitor asymmetric dimethylarginine.³⁹

In a cohort of patients with coronary artery disease undergoing bypass graft surgery, high plasma BH4 concentrations were associated with impaired acetylcholine-induced vasorelaxation in saphenous vein rings, whereas high tissue BH4 concentrations were associated with greater relaxation.⁴⁰ These findings suggest an inverse relationship between plasma BH4 and vascular function, but these effects may be independently driven by inflammation as opposed to functionally related.⁴⁰ Rodent erythrocytes express GTPCH during development, though activity is much lower in fully mature erythrocytes.⁴¹ *P. berghei* ANKA parasites preferentially infect young erythrocytes and reticulocytes⁴² and may specifically disrupt BH4 synthesis in the cell population with the highest GTPCH activity. An atypical pyruvoyl tetrahydropterin synthase enzyme is expressed by *P. falciparum* that is capable of utilizing the BH4 biosynthesis intermediate dihydroneopterin triphosphate for folate synthesis.⁴³ If *P. berghei* ANKA parasites express a similar enzyme, then the utilization of dihydroneopterin triphosphate for folate synthesis instead of BH4 synthesis may contribute to BH4 depletion.⁴³ It has been recently suggested that erythrocytes contain functional eNOS,^{44–46} although others have found no evidence of erythrocyte eNOS activity.⁴⁷ A variety of NOS inhibitors have also been shown to reduce red blood cell deformability,^{44,48} although the mechanism by which NO may maintain deformability has yet to be determined. It is therefore possible that BH4 depletion could contribute to the poor erythrocyte deformability, impaired microvascular perfusion, insufficient oxygen delivery, and acidosis observed in patients with severe malaria.^{49,50}

In contrast to plasma, erythrocytes, and brain tissue where BH4 levels were lower, BH4 levels were higher in the aorta and liver of mice infected with *P. berghei* ANKA. Pro-inflammatory signaling results in the upregulation of GTP cyclohydrolase transcription and BH4 synthesis in endothelial cells⁵¹ and may contribute to elevated BH4 observed in aorta and liver tissue. Phenylalanine also induces BH4 synthesis.⁵² Phenylalanine accumulation in liver tissue could have contributed to elevated BH4 levels there, but in the brain,

phenylalanine elevation was associated with low BH4 in infected mice. Taken together, these results suggest that different degrees or types of inflammation may account for the rise in aortic and liver BH4 in contrast to the decrease in BH4 observed in brain tissue from infected mice.

Our findings provide novel support for citrulline infusion as a viable approach to restoring NOS substrate availability in severe malaria. In our studies, citrulline infusion supplemented plasma pools of both citrulline and arginine. These findings indicate effective arginine synthesis by argininosuccinate synthase 1 (ASS1) and argininosuccinate lyase (ASSL) during *P. berghei* ANKA infection. Recent studies have shown that ASS1 and ASL complex with NOS,⁵³ raising the possibility that citrulline could serve as an important substrate pool via rapid recycling to arginine directly prior to utilization by NOS.^{54,55} In support of this mechanism, citrulline is a more effective substrate for NO synthesis in macrophages with mycobacterium-induced arginase expression⁵⁶ comparable to recent reports of monocyte arginase induction in severe malaria.⁵⁷ Thus, in addition to serving as a precursor for systemic arginine pools citrulline may serve as an NOS substrate that is protected from extracellular or intracellular arginase.^{8,57}

Our study addressed the question of whether the concomitant supplementation of arginine and BH4 could restore NOS activity. Under conditions of low BH4, increasing concentrations of arginine accelerate NADPH consumption and increase superoxide production by uncoupled eNOS.⁵⁸ Therefore, the restoration of arginine without correcting the BH4 deficiency may be ineffective or even harmful. This mechanism may explain why clinical studies of arginine supplementation have predominantly demonstrated a lack of efficacy or even harm in disease states associated with BH4 depletion. For example, arginine supplementation has been shown to worsen outcomes in patients with peripheral artery disease⁵⁹ or myocardial infarction.⁶⁰

Our study includes several limitations. First and foremost, the use of continuous infusion in a mouse model significantly limited the overall number of control and experimental animals that could be included in our experimental design. We elected to focus on the most critical control groups (uninfected + saline and infected + saline) and an experimental group (infected + sepiapterin/citrulline) that would allow us to assess the impact of BH4 and arginine supplementation on PAH and NOS activity. Further experimentation is required to hone the impact of either sepiapterin or citrulline alone or to compare the efficacy of citrulline to an equivalent dose of arginine or ornithine. Second, infected mice infused with sepiapterin and citrulline consumed more food than infected controls infused with saline on day 6 of infection. While this may reflect a modest improvement in clinical status, we cannot rule out the possibility that increased dietary nitrite intake raised the concentration of blood nitrite in the treated animals. Third, the BH4 concentrations we observed in murine plasma and erythrocytes were much higher than those previously reported in humans. For example, in a study of patients with coronary artery disease, plasma BH4 concentrations were 21.3 [11.5–42.1] nmol/L (median [IQR]) and plasma 7,8-BH2 concentrations were 15.1 [11.6–19.2] nmol/L.⁴⁰ Murine BH4 concentrations in our experiment also exceeded published human erythrocyte BH4 concentrations of 14.5 ± 8.4 nmol/L (mean $\pm$ SD) in children and 12.5 ± 7.1 nmol/L in (mean $\pm$ SD) in adults.⁴¹ Internal mammary artery and

saphenous vein BH4 concentrations have been reported in pmol/g of tissue,⁴⁰ limiting comparison to our measurements in murine tissue that were normalized to soluble protein. Finally, it is difficult to generalize data from the *P. berghei* ANKA model to human cerebral malaria because of distinct differences in pathophysiology and the poor preclinical predictive value for experimental interventions.⁶² However, we also observe that the *P. berghei* ANKA model replicates clinical observations of arginine depletion^{8,33} and phenylalanine elevation.⁷ We therefore conclude that the *P. berghei* ANKA model is a useful tool for the investigation of specific biochemical pathways but that findings should ultimately be validated in clinical studies of human patients.

In summary, we successfully raised both BH4 and arginine in *P. berghei*-infected mice via infusion of the precursors sepiapterin and citrulline. This improved phenylalanine metabolism and nitric oxide synthesis but failed to improve behavioral symptoms in *P. berghei* ANKA-infected mice. The failure of sepiapterin and citrulline infusion to significantly improve behavioral scores should not preclude the further investigation of these agents in patients with severe malaria. Our results suggest that current investigations of intravenous arginine as a therapy for severe malaria^{63–65} would benefit from a consideration of citrulline infusion as an alternate approach to restoring arginine availability. Citrulline infusion may circumvent the elevation of plasma⁸ or intracellular⁵⁷ arginase activity by delivering an effective substrate directly to the ASS1/ASL/NOS complex. Further investigation of arginine restoration should also consider sepiapterin cosupplementation, which we have identified as an effective method of improving BH4 availability in both blood and tissue compartments. Although sepiapterin treatment may not be sufficient to restore BH4/7,8-BH2 ratios in plasma or brain tissue, our results suggest that sepiapterin effectively improved phenylalanine hydroxylase activity. These results provide a compelling case to investigate whether sepiapterin treatment and the normalization of phenylalanine metabolism in patients with severe malaria may improve outcomes.

INNOVATION

Hyperphenylalaninemia and impaired endothelium-dependent vasodilation are thought to contribute to the pathogenesis of severe malaria infection. Using a mouse model of severe malaria, we identified blood and tissue depletion of tetrahydrobiopterin (BH4), an oxidant-sensitive cofactor required by both phenylalanine hydroxylase (PAH) and nitric oxide synthase (NOS). We found that the infusion of sepiapterin (a BH4 precursor) and citrulline (an arginine precursor) improved phenylalanine metabolism and NO synthesis. Our findings suggest that BH4 depletion is a common biochemical mechanism of PAH and NO synthase impairment that could be targeted therapeutically in patients with severe malaria.

METHODS

Experimental Animals.

Animal experiments were performed at the National Institutes of Health (NIAID Comparative Medicine Branch) using protocols approved by the NIH ACUC under identification ASP LMVR 18E. Ten-week-old male C57BL/6J mice were purchased with surgically implanted silicone jugular vein catheters (2 Fr at the insertion point, 3 Fr at the

access point) from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in individual cages specialized to support free movement of the tether system (Instech Solomon, Plymouth Meeting, PA, USA). Individual cages were fitted with removable water bottles and food hoppers. All mice were maintained at 20–22 °C with a 12 h/12 h light–dark cycle and free access to water and autoclaved rodent feed pellets (Teklad Global 18% protein extruded rodent diet, 2018SX, Harlan Laboratories, Frederick, MD, USA). Ten-week-old Swiss Webster carriers for parasite expansion were obtained from Charles River Laboratories (Frederick, MD, USA).

Infections.

P. berghei ANKA parasites, clone RMgm-29,⁶⁶ were expanded from frozen stocks by intraperitoneal injection of carrier mice. After 4 days of parasite expansion, whole blood was obtained from the abdominal inferior vena cava under terminal anesthesia. Cohorts of experimental mice were infected by the intraperitoneal injection of 1×10^6 infected erythrocytes diluted in 200 μL of PBS (Lonza, Walkersville, MD, USA). This protocol results in blood-stage infection, bypassing the liver stage of the parasite's lifecycle.

Infusion of Sepiapterin and Citrulline.

Mice were anesthetized with inhaled isoflurane (4% induction, 2% maintenance) during setup and connection of the infusion line. The catheter was secured in place with a specialized harness (Instech Solomon, Plymouth Meeting, PA, USA) and attached to an infusion line supported by a tether system with a spring-balanced arm and swivel (Instech Solomon, Plymouth Meeting, PA, USA).

Sepiapterin (Schirck's Laboratories, Jona, Switzerland) and citrulline (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 0.9% sterile saline (Hospira, Lake Forest, IL, USA), sterilized with a 0.22 μm syringe-tip filter, divided into aliquots, and stored at -80°C until use. Concentrations of sepiapterin and citrulline were selected to provide doses of 15 $\text{mg kg}^{-1} \text{ day}^{-1}$ and 2.4 $\text{g kg}^{-1} \text{ day}^{-1}$ respectively, delivered at a rate of 25 $\mu\text{L/h}$. Uninfected and infected control mice were infused with 0.9% sterile saline (Hospira, Lake Forest, IL, USA). All infusions were delivered with a PHD2000 syringe pump (Harvard Apparatus, Holliston, MA, USA). Infusions were started the day before inoculation and continued until the mice were sacrificed on day 6 postinoculation.

Behavioral Scoring and Quantification of Parasitemia.

Behavioral symptoms were scored using a quantitative scoring scale adapted from the rapid murine coma and behavior scale.⁶⁷ Scores were assigned on a scale of 0 (moribund) to 2 (normal) for each of seven indicators: gait, balance, exploratory activity, body position, limb strength, touch escape, and pinna reflex. Parasitemia was monitored by an examination of Giemsa-stained blood smears obtained by a tail tip bleed in live animals or from blood samples taken during tissue collection.

Blood and Tissue Collection.

Terminal anesthesia was administered by the intraperitoneal injection of 250 mg/kg ketamine and 25 mg/kg xylazine. The abdominal cavity was accessed, and 700 μL of blood

was drawn from the abdominal inferior vena cava with a 25G $\times$ 5/8" needle (BD, Franklin Lakes, NJ, USA). To prevent coagulation, the syringe contained 60 μ L of DPBS with K₃EDTA (final concentration of 1.6 mg/mL K₃EDTA after 700 μ L of blood drawn). Plasma was separated from erythrocytes by centrifugation at 3000g for 7 min at 4 °C and snap-frozen on dry ice. The buffy coat was removed and discarded to ensure the purity of red blood cell samples. Mice were transcardially perfused with 20 mL of PBS, and the lung, liver, kidney, spleen, aorta, heart, and whole brain were dissected, removed, and snap-frozen on dry ice. All samples were stored at –80 °C until analysis.

HPLC Analysis of Amino Acids in Plasma and Tissue.

Plasma samples were deproteinated by mixing 12 μ L of sample with 12 μ L of 2 M perchloric acid (HClO₄, Fisher Scientific, Pittsburgh, PA, USA) containing 50 μ M L-norvaline as an internal standard (Sigma-Aldrich, St. Louis, MO, USA). After centrifuging for 5 min at 16100g and 4 °C, supernatants were removed and neutralized by adding 10 μ L to 5 μ L of 1.5 M potassium carbonate (Sigma-Aldrich, St. Louis, MO, USA) in a fresh tube. The resulting solution was diluted 1:2 in ultrapure water from a Milli-Q synthesis system (EMD Millipore, Billerica, MA, USA) to a final dilution of 1:6. After centrifuging for 5 min at 16100g and 4 °C to remove potassium chlorate precipitate, samples were loaded into screw-cap polypropylene vials (Agilent Technologies, Santa Clara, CA, USA) for analysis. Amino acid standards (Sigma-Aldrich, St. Louis, MO, USA) were diluted to 50 μ M and processed in parallel to serve as an external standard for quantitation. HPLC was performed on a low-flow Agilent 1100 series instrument, and Agilent Chemstation OpenLab CDS revision C.01.03 was used to control the instrument and acquire data. Samples were derivatized immediately prior to analysis with 1.5 mg/mL (11.2 mM) *ortho*-phthaldialdehyde (Sigma-Aldrich, St. Louis, MO, USA) and 41.3 mM 3-mercaptopropionic acid (Sigma-Aldrich, St. Louis, MO, USA) diluted in 200 mM potassium tetraborate solution (pH 9.4).

Analytes were separated with a 1.0 mm $\times$ 150 mm Luna C18(2) column with 5 μ m particles and 100 Å pore size (Phenomenex, Torrance, CA, USA) protected with a 1.0 mm $\times$ 13 mm ACE C18 guard column with 3 μ m particles and 100 Å pore size (MAC-MOD Analytical, Chadds Ford, PA, USA). The aqueous mobile phase (solvent A) consisted of 0.1 M sodium acetate (Fisher Scientific, Pittsburgh, PA, USA) in ultrapure H₂O from a Milli-Q synthesis system (Millipore, Billerica, MA, USA) with 9% v/v methanol (Fisher Scientific, Pittsburgh, PA, USA) and 0.5% v/v tetrahydrofuran (Sigma-Aldrich, St. Louis, MO, USA) with the pH adjusted to 8.3 with 3 M NaOH (Sigma-Aldrich, St. Louis, MO, USA). The organic mobile phase (solvent B) was HPLC-grade methanol (Fisher Scientific, Pittsburgh, PA, USA). Flow rates and solvent ratios were as follows: 0 min, 96% A; 10.87 min, 86% A; 16.3 min, 86% A; 21.73 min, 70% A; 27.17 min, 60% A; 28.8 min, 53% A; 35.32 min, 50% A; 40.75 min, 30% A; 43.5 min, 0% A; 48.5 min, 0% A; 48.6 min, 96% A; and 65 min, 96% A. The column was maintained at 40 °C for the duration of the run. Online fluorescence was measured at excitation and emission wavelengths of 340 and 455 nm, respectively. Concentrations were calculated using both internal (L-norvaline) and external standards. Tissue samples (~30 mg) were homogenized in 1X PBS (Lonza, Walkersville, MD, USA) using a hand-held homogenizer (Cole Parmer, Vernon Hills, IL, USA), centrifuged for 5 min

at 16100g and 4 °C to remove insoluble protein, and processed as described above. Tissue amino acid concentrations were normalized to soluble protein content as determined by the Pierce BCA protein assay (Thermo Scientific, Sunnyvale, CA, USA) according to the manufacturer's instructions.

HPLC Determination of BH4 and 7,8-BH2.

BH4 and 7,8-BH2 concentrations in tissue, plasma, and red blood samples were determined by high-performance liquid chromatography (HPLC) with sequential electrochemical and fluorescence detection. Tissue samples (~30 mg) were homogenized in 15 μ L of ice-cold resuspension buffer per milligram (~450 μ L) using a hand-held homogenizer (Cole Parmer, Vernon Hills, IL, USA). The resuspension buffer consisted of 1 mM dithioerythritol (Acros Organics, Geel, Belgium) and 0.5 μ M EDTA (Sigma-Aldrich, St. Louis, MO, USA) diluted in 1X DPBS (Lonza, Walkersville, MD, USA). Homogenized samples were centrifuged for 5 min at 16100g and 4 °C to remove insoluble protein. To precipitate proteins, supernatants were then mixed 9:1 with ice-cold precipitation acid consisting of 1 M phosphoric acid (Fisher Scientific, Pittsburgh, PA, USA), 2 M trichloroacetic acid (Fisher Scientific, Pittsburgh, PA, USA), and 1 mM dithioerythritol (DTE) (Acros Organics, Geel, Belgium) diluted in 1X DPBS (Lonza, Walkersville, MD, USA). Instead of homogenization, whole aortas were subjected to three freeze-thaw cycles on dry ice in 200 μ L of resuspension buffer prior to mixing with precipitation acid as above. Red blood cells and plasma samples were diluted 1:10 in resuspension buffer prior to mixing with precipitation acid as above. Following precipitation, all samples were centrifuged for 5 min at 16100g and 4 °C. Supernatants were loaded into 250 μ L capacity polypropylene vials (Agilent Technologies, Santa Clara, CA, USA) and loaded into the autosampler. All samples were maintained on ice during processing.

Samples (100 μ L) were injected onto an isocratic HPLC system consisting of an 1100 series degasser and an isocratic pump and a 1260 Infinity series autosampler, temperature-control unit, and fluorescence detector with an 8 μ L flow cell, all obtained from Agilent Technologies (Santa Clara, CA, USA). A CouloChem III electrochemical detector (Thermo Scientific, Sunnyvale, CA, USA) was installed after the column and before the fluorescence detector. Chromatography was performed on a 4.6 mm $\times$ 250 mm ACE C18 column with 5 μ m particles and a 100 Å pore size (MAC-MOD Analytical, Chadds Ford, PA, USA) with a 4.6 mm $\times$ 13 mm ACE C18 guard column with 5 μ m particles and 100 Å pore size (MAC-MOD Analytical, Chadds Ford, PA, USA). Analytes were separated isocratically with a mobile phase composed of 50 mM HPLC-grade sodium acetate (Fisher Scientific, Pittsburgh, PA, USA), 5 mM citric acid (Fisher Scientific, Pittsburgh, PA, USA), 48 μ M EDTA (Sigma-Aldrich, St. Louis, MO, USA), and 160 μ mol/L dithioerythritol (DTE) (Acros Organics, Geel, Belgium) prepared in ultrapure water (Milli-Q synthesis system, Millipore Corporation, Billerica, MA, USA). The flow rate was 1.3 mL/min with a 19 min run time between sample injections. For the detection of BH4, the electrochemical detector's analytical cell was set to a voltage of +75 mV for electrode 1 and -400 mV for electrode 2. The guard cell voltage was set to +900 mV to oxidize 7,8-BH2 to biopterin, which is fluorescent. Oxidized 7,8-BH2 was quantified using fluorescence detector excitation and

emission wavelengths of 350 and 450 nm, respectively, and a photomultiplier tube gain of 18.

BH4 and 7,8-BH2 were quantified by the comparison of integrated peak areas to external standard solutions of BH4 and 7,8-BH2 (Schircks Laboratories, Jona, Switzerland) diluted to known concentrations (0.1–50 nmol/L). For tissue analysis, measured concentrations were normalized to soluble protein content, which was separately assayed with a Pierce BCA protein assay kit (Thermo Scientific, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Whole Blood Nitrite.

Immediately after collection, 100 μ L of whole blood was mixed with 25 μ L of a solution containing potassium hexacyanoferrate(III) (800 mM) (Sigma-Aldrich, St. Louis, MO, USA) to oxidize ferrous heme, *N*-ethylmaleimide (100 mM) (Sigma-Aldrich, St. Louis, MO, USA) to block thiols, and 10% v/v Nonidet-40 substitute (Fisher Scientific, Pittsburgh, PA, USA) to solubilize cell membranes. Samples were promptly snap-frozen on dry ice and stored at -80°C until analysis. For analysis, whole blood samples were thawed and deproteinated by mixing 1:1 with methanol (EMD Millipore, Billerica, MA, USA) and centrifuging for 5 min at 16100g and 4°C . Supernatants were injected into a reaction chamber containing potassium iodide (66.9 mM) and iodine (28.5 mM) (Sigma-Aldrich, St. Louis, MO, USA) diluted in a 2:7 mixture of ultrapure water (Milli-Q Synthesis System, Millipore Corporation, Billerica, MA, USA) and glacial acetic acid (Fisher Scientific, Pittsburgh, PA, USA). NO produced by the reduction of nitrite in plasma samples was detected with a nitric oxide analyzer 280i (GE Healthcare, Pittsburgh, PA, USA) and compared with standards of known concentration prepared from sodium nitrite (Sigma-Aldrich, St. Louis, MO, USA). Data processing and peak integrations were performed with OriginPro 8 software.

Statistical Analyses.

Data are presented in the median and interquartile range (IQR). Statistical analyses were performed with GraphPad Prism 6 software, and $p < 0.05$ was considered to be significant. The Mann–Whitney test was used to compare groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BH2	7,8-dihydrobiopterin
BH4	tetrahydrobiopterin
NOS	nitric oxide synthase
PAH	phenylalanine hydroxylase

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Figure 1.

Tetrahydrobiopterin synthesis, utilization, and recycling. Tetrahydrobiopterin (BH₄) is an essential cofactor in the conversion of phenylalanine to tyrosine and in the synthesis of nitric oxide (NO) from arginine and oxygen. Tetrahydrobiopterin is synthesized de novo from GTP or recycled from dihydrobiopterin (BH₂) by reactions that require reducing equivalents provided by NADH or NADPH. Thus, phenylalanine metabolism and NO synthesis are partially dependent on the redox status of the cell. GTP, guanosine triphosphate; GTPCH, GTP cyclohydrolase; DNTP, dihydroneopterin triphosphate; PTPS, pyruvoyl tetrahydropterin synthase; 6-PTP, 6-pyruvoyl-tetrahydropter-in; SR, sepiapterin reductase; BH₄, tetrahydrobiopterin; PAH, phenylalanine hydroxylase; q-BH₂, quinonoid-dihydrobiopterin; DHPR, dihydropteridine reductase; 7,8-BH₂, 7,8-dihydrobiopterin; DHFR, dihydrofolate reductase; NOS, nitric oxide synthase; and NO, nitric oxide.

Figure 2.

P. berghei infection depletes BH4 and lowers the BH4/BH2 ratio in plasma, red blood cells, and brain tissue. Plasma, erythrocyte, and tissues BH4 and 7,8-BH2 were determined by HPLC on day 6 postinoculation. *P. berghei* ANKA-infected mice received a continuous intravenous infusion of saline ($n = 9$) or $2.4 \text{ g kg}^{-1} \text{ day}^{-1}$ citrulline + $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ sepiapterin ($n = 11$) from day -1 to day 6 postinoculation. Tissue concentrations were normalized to soluble protein. Saline-infused uninfected mice ($n = 12$) served as controls. Data are pooled from three independent experiments. Box plots depict the median and IQR. Whiskers extend to the highest and lowest values within 1.5IQR of the 75th and 25th percentiles, and values outside this range were plotted as individual points (Tukey's method). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by the Mann-Whitney test. ns (not significant): $p > 0.05$.

Figure 3.

Infusion of sepiapterin and citrulline restores plasma, liver, and brain phenylalanine/tyrosine ratios. Plasma and tissue phenylalanine and tyrosine were determined by HPLC 6 days postinoculation with *P. berghei* ANKA. Tissue concentrations were normalized to soluble protein. *P. berghei* ANKA-infected mice received a continuous intravenous infusion of saline ($n = 9$) or $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ sepiapterin + $2.4 \text{ g kg}^{-1} \text{ day}^{-1}$ citrulline ($n = 11$) from day -1 to day 6 postinoculation. Saline-infused uninfected mice ($n = 12$) served as controls. Data are pooled from three independent experiments. Box plots depict the median, IQR, min, and max. Whiskers extend to the highest and lowest values within 1.5IQR of the 75th and 25th percentiles, and values outside this range were plotted as individual points (Tukey's method). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by the Mann–Whitney test. ns (not significant): $p > 0.05$.

P. berghei infection lowers the arginine bioavailability in plasma but not in tissue. Plasma and tissue arginine, citrulline, and ornithine were determined by HPLC on day 6 postinoculation with *P. berghei* ANKA. Tissue amino acid concentrations were normalized to soluble protein. *P. berghei* ANKA-infected mice received a continuous intravenous infusion of saline ($n = 9$) or $2.4 \text{ g kg}^{-1} \text{ day}^{-1}$ citrulline + $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ sepiapterin ($n = 11$) from day -1 to day 6 postinoculation. Saline-infused uninfected mice ($n = 12$) served as controls. Data are pooled from three independent experiments. Citrulline in brain tissue was not determined (ND) because the peak was too small and too poorly resolved from a closely eluting unidentified peak for reliable quantification. Box plots depict the median and IQR. Whiskers extend to the highest and lowest values within 1.5IQR of the 75th and 25th percentiles, and values outside this range were plotted as individual points (Tukey's method). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ by the Mann–Whitney test. ns (not significant): $p > 0.05$.

Figure 5.

Infusion of sepiapterin and citrulline improves whole blood nitrite in *P. berghei* ANKA-infected mice. *P. berghei* ANKA-infected mice received a continuous intravenous infusion of saline ($n = 9$) or $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ sepiapterin + $2.4 \text{ g kg}^{-1} \text{ day}^{-1}$ citrulline ($n = 11$) from day -1 to day 6 postinoculation. Whole blood samples were collected on day 6 postinoculation. Saline-infused uninfected mice ($n = 12$) served as controls. Data are pooled from three independent experiments. Box plots depict the median, IQR, and range. * $p < 0.05$ by the Mann–Whitney test.

Figure 6.

Effect of sepiapterin and citrulline infusion on the behavior of infected mice. *P. berghei* ANKA-infected mice received a continuous intravenous infusion of saline ($n = 15$) or $15 \text{ mg kg}^{-1} \text{ day}^{-1}$ sepiapterin + $2.4 \text{ g kg}^{-1} \text{ day}^{-1}$ citrulline ($n = 16$) from day -1 to day 6 postinoculation. Saline-infused uninfected mice ($n = 15$) served as controls. Symptoms were scored by assessing each of seven parameters on a scale of 0 (moribund) to 2 (normal). D6 (AM) scores reflect assessments taken from 7:00 a.m. to 9:00 a.m. for all animals, and D6 (Cull) scores reflect assessments taken directly before each animal was culled for tissue collection. Pooled data from four independent experiments are plotted as individual points with medians indicated by black lines. *** $p < 0.001$ by Mann–Whitney test.