Functional Tetrahydrobiopterin Synthesis in Human Platelets

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Background—Previous studies have provided evidence for the importance of platelet-derived nitric oxide (NO) for the regulation of hemostasis. Tetrahydrobiopterin (BH₄) is an essential cofactor and regulator of NO synthase activity in the vasculature; however, it is as yet unknown whether platelets dispose over a functional BH₄ synthesis.

Methods and Results—We quantified mRNA expression of genes involved in BH₄ synthesis, measured enzymatic activities, and determined intraplatelet levels of pteridines in platelets from healthy volunteers and from patients treated for prolonged periods of time with glucocorticoids. Freshly isolated platelets from healthy volunteers show functional BH₄ synthesis, as evidenced by the presence of mRNA species and enzymatic activity of GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase. Biopterin was the major intraplatelet pteridine, whereas no neopterin was found. mRNA expression and enzymatic activity of GTPCH were undetectably low in platelets that had been stored for 5 days, and no pteridines were found in these platelets. Freshly isolated platelets from patients treated with glucocorticoids had decreased mRNA expression and activity of GTPCH compared with platelets from healthy volunteers.

Conclusions—Human platelets dispose over a functional de novo BH₄ synthesis. Furthermore, our results indicate the potential of external factors, eg, prolonged storage or glucocorticoid therapy, to significantly affect BH₄ synthesis within platelets. Together, these findings offer new insights into the biology and pathobiology of platelet function in humans. (Circulation. 2004;110:186-192.)

Key Words: tetrahydrobiopterin ▪ platelets ▪ nitric oxide synthase

Nitric oxide (NO) plays a pivotal role in the vascular system ¹: It not only regulates vessel wall tone but also affects the hemostatic-thrombosis balance, eg, by potently inhibiting platelet activation and aggregation. ¹⁻³ Under normal conditions, the major source of NO is the endothelium, which produces NO by means of a constitutive NO synthase (cNOS). ¹ However, expression and functional activity of cNOS have also been found in human platelets, and regulation of hemostasis by platelet-derived NO has been well documented. ³⁻⁷

Tetrahydrobiopterin (BH₄) is an essential cofactor and regulator of NOS activity in the vascular system. ⁸ Recently, results from a canine thrombosis model suggested an important role of intraplatelet BH₄ for the regulation of coronary arterial thrombosis by modulating platelet-derived NO production. ⁹ However, it is as yet unknown whether BH₄ stems from extraplatelet sources, eg, the endothelium, ⁸ or whether platelets themselves dispose over a functional BH₄ de novo biosynthesis, a complex multistep process. ⁸,¹⁰ Briefly, BH₄ synthesis starts from GTP, which is converted to the first intermediate, 7,8-dihydroneopterin triphosphate, by GTP-cyclohydrolase I (GTPCH). The first intermediate is then converted by 6-pyruvoyl tetrahydropterin synthase (PTPS) and subsequently by sepiapterin reductase (SR) to the end product, BH₄ (Figure 1). In the present study, using molecular and biochemical methods, we investigated whether the BH₄ de novo biosynthesis pathway is functional in human platelets. To gain preliminary insights into potential influences and regulatory mechanisms, we analyzed BH₄ synthesis in freshly isolated platelets from healthy donors and in platelets from healthy donors after prolonged storage. In addition, we analyzed BH₄ synthesis in platelets from patients treated with glucocorticoids and from patients with sepsis or septic shock conditions, known to significantly affect extraplatelet BH₄ synthesis. ⁸,¹⁰

Methods

Source of Platelets

Unless otherwise stated, platelets were used from 11 healthy white hospital employees (7 men, 4 women; median age, 32 years; range,
25 to 60 years). They did not take any medication, especially anticoagulants, steroids, or nonsteroidal antiinflammatory drugs. Platelet concentrates, which were stored for 5 days, were obtained from the University Hospital Blood Transfusion Unit, Zürich. To study the influence of glucocorticoids on BH4 synthesis, we used blood of patients treated with glucocorticoids in a dose equivalent to at least 100 mg of prednisone for at least 7 days. These 6 patients were all white, 3 men and 3 women, with a median age of 51.5 years (range, 24 to 79 years). They did not receive anticoagulants, were white, 3 men and 3 women, with a median age of 54 years (range, 50 to 68 years). The most frequent indication for glucocorticoids was arthritis, 50 to 68 years). To study the influence of inflammatory stimuli on BH4 synthesis, platelets were isolated from blood of patients fulfilling the criteria of sepsis or septic shock.11 These 4 patients were white, 2 men and 2 women, with a median age of 51.5 years (range, 24 to 79 years). They did not receive anticoagulants, glucocorticoids, or nonsteroidal antiinflammatory drugs and were not affected by HIV. Blood was drawn within 48 hours of initial diagnosis of sepsis or septic shock, and all patients had severe signs of systemic inflammation (mean CRP value, 260 ± 116 mg/L; nor-

RNA Isolation and Quantitative Real-Time RT-PCR

Total RNA was isolated by use of the Qiagen RNeasy Mini Kit (Qiagen) including a DNAse digestion. RNA was quantified photometrically and used directly or stored at −70°C. Human coronary artery endothelial cells (HCAECs) were used as positive control. Culture of HCAECs and preparation of HCAEC mRNA was performed as described previously.12 Total platelet RNA (200 ng) was reverse-transcribed (RT) into cDNA with the ProSTAR First Strand Kit (Stratagene). cDNA was amplified by real-time polymerase chain reaction (PCR) with the Light Cycler using the Fast Start DNA Master SYBR Green I (both Roche Diagnostic). Cycling conditions were as follows: initial denaturation (10 minutes, 95°C), followed by 45 cycles of denaturation (15 seconds, 95°C), annealing (10 seconds, 55°C to 67°C), and extension (12 seconds, 72°C), with acquisition of fluorescence at the end of each extension. PCR products were labeled with SYBR Green I, which fluoresces only when bound to double-stranded DNA. Real-time PCR results were analyzed with the Light Cycler analysis software version 3.5. Sequence-specific PCR primers used were as follows: for GTPCH, 5′-TTGGTTATCTTCTTCTAACAAG3′ (forward) and 5′-GTGCCTGTCAGACAGTTG3′ (reverse); for PTPS, 5′-CGAGCCACCGATTGCTACGAA3′ (forward) and 5′-GTGTCGCCAGATAAACGCTACA3′ (reverse); for SR, 5′-CTGTTCCAGTATAAACGCTACA3′ (reverse) or water (negative control), respectively. Amplicon specificity was verified by sequencing. Quantitative analysis of mRNA species was performed as described previously.13 Values of mRNA species were corrected with the corresponding values for the housekeeping gene GAPDH. The detection threshold was set at 35 cycles. One arbitrary unit (AU) was defined as 35 cycles corresponding to E−30cp, with E defined as PCR efficiency and determined from the slope of the standard curve as E = 10−1/cp.14,15

Pteridine Profiles

Platelets were washed twice with PBS (Gibco) and lysed in 200 μL of 0.1 mol/L HCl by freezing in liquid nitrogen/thawing. Pteridines in platelet lysates and plasma from healthy donors were measured by high-performance liquid chromatography (HPLC) after acidic oxidation with MnO2 as described previously.16

Enzyme Activity Assays

Activity assays for BH4 synthesis enzymes were performed in lysates of platelets isolated from 50 mL of citrated blood, which typically yielded ≈ 2 to 3.6 × 109 platelets. Platelet pellets were washed twice with PBS, and assays for GTPCH, PTPS, and SR activity were performed according to validated diagnostic protocols.17 By definition, 1 unit of GTPCH produces 1 μmol of neopterin per minute, 1
unit of PTPS produces 1 μmol of bioperin per minute, and 1 unit of SR produces 1 μmol of bioperin per minute at 37°C.

Statistical Analysis
Results are expressed as mean±SEM for data from several independent experiments or mean±SD for data from 1 representative experiment with duplicate measurements. Data were analyzed with unpaired 2-tailed Student’s t test or 1-way ANOVA using Instat 3.0 (GraphPad Software). A value of P<0.05 was considered statistically significant.

Results
BH₄ Synthesis Is Functional in Normal Platelets
In a first series of experiments, we measured mRNA expression and enzyme activity of GTPCH, PTPS, SR, and cNOS, as well as the presence of pteridin metabolites, in platelets freshly isolated from healthy volunteers. As shown in Figure 2, we were able to detect mRNA expression for all enzymes required for BH₄ biosynthesis in significant abundance by real-time RT-PCR. Using AU as defined in the Methods section and normalized for 10¹⁰ platelets, we measured 25.91±11.31 AU for GTPCH, 14.65±10.52 AU for PTPS, and 13.1±4.27 AU for SR (mean±SEM, n=4), respectively. Comparison with transcript levels found in HCAECs, cells with known expression levels of these genes, revealed a similar expression pattern of these genes; however, expression levels were consistently lower in platelets compared with HCAECs, as evidenced by the amplification curves (Figure 2, left). In contrast to the expression of GTPCH, PTPS, and SR, mRNA expression of cNOS was only minimal in human platelets, whereas it could easily be detected in HCAECs (see Figure 2).

Next, enzyme activity assays were performed to verify that all 3 enzymes of the de novo BH₄ synthesis were functional in platelets. Indeed, we measured activities for GTPCH of 3.5±1.4 μU/10¹⁰ platelets, and for PTPS of 5.3±0.7 μU/10¹⁰ platelets (data are expressed as mean±SD of duplicate measurements from 1 representative experiment). An ≈10 000-fold higher SR activity of 461±36 mU/10¹⁰ platelets was detected in human platelets, a value of SR activity that is comparable to values found in other human cell types.

Finally, as shown in Figure 3, HPLC analysis of intracellular pteridines after acidic oxidation of lysates from freshly prepared platelets revealed the presence of biopterin, the final oxidation product of BH₄, and dihydrobiopterin, and pterin, a degradative oxidation product of BH₄. In contrast, no neopterin, a side-product of the first intermediate of BH₄ synthesis (see Figure 1), was detectable in platelets. A total of 10¹⁰ platelets contained 18.3±1.5 pmol of total biopterin and 10.3±1.2 pmol pterin (n=3) (data are mean±SEM of duplicate measurements from 3 independent experiments). By comparison, a typical profile from human plasma from healthy volunteers revealed the presence of significant amounts of neopterin, little biopterin, and no pterin (Figure 3B).

BH₄ Biosynthesis Is Abolished After Prolonged Storage of Platelets
To investigate the effect of platelet storage on the BH₄ synthesis pathway, we next examined platelets from healthy donors that were stored under standardized conditions for 5 days in plasma for the presence of the BH₄ synthetic enzymes and metabolites. In contrast to the results obtained in freshly isolated platelets, GTPCH mRNA was no longer detectable in stored platelets, whereas PTPS and SR mRNA expression was ≈500-fold and 10-fold lower compared with freshly isolated platelets, respectively (Figure 4). GTPCH enzymatic activity was no longer detectable in stored platelets, and accordingly, stored platelets did not contain any measurable amounts of pteridines (Figure 3C). However, minimal cNOS mRNA expression was still detected in platelets that were stored for 5 days, and expression levels in these platelets did not differ significantly from levels found in freshly isolated platelets (not shown).

GTPCH Gene Expression Is Inhibited by Glucocorticoids
In other human cell types, GTPCH is regulated by glucocorticoids and inflammatory stimuli, which result in reduced or enhanced expression, respectively. Thus, we investigated whether such regulatory mechanisms also influence the activity of BH₄ synthesis in human platelets, specifically whether glucocorticoid treatment downregulates GTPCH mRNA expression. It has to be noted, however, that platelets are anucleated fragments from megakaryocytes and therefore direct expressional gene regulation does not take place in platelets but only during megakaryocyte development in the bone marrow. Taking the half-life of platelets into consideration, we therefore studied expression of genes involved in BH₄ synthesis in platelets of patients who were treated with significant doses of glucocorticoids for at least 7 days. As shown in Figure 4, a reduced expression of GTPCH mRNA was observed in platelets of patients treated with steroids compared with platelets that were freshly isolated from healthy volunteers (P<0.0001). In contrast, no difference in PTPS and SR mRNA expression was found in patients treated with glucocorticoids.

Discussion
The present study reveals 3 major novel findings: first, our data clearly demonstrate that platelets from healthy adult volunteers possess a functional de novo BH₄ biosynthesis; second, prolonged storage of platelets from healthy volunteers results in abrogation of intrinsic BH₄ synthesis; and, third, expression of genes involved in BH₄ synthesis might be affected by pathological conditions, eg, steroid therapy. Using freshly isolated platelets obtained from healthy volunteers, we were able to show the presence of mRNA species of all genes involved in de novo BH₄ biosynthesis in significant abundance. Moreover, measurement of enzymatic activities unequivocally demonstrates the functionality of the BH₄ biosynthesis pathway. Measurement of intraplatelet pteridin levels shows a distinct pattern of abundance, with relatively high levels of biopterin, lower levels of the oxidation product pterin, and no neopterin. It is highly unlikely that contamination of blood components other than platelets might account for the present findings. Most importantly, cell contamination of all platelet preparations was excluded morphologically by Coulter counter analysis. Furthermore, the pteridine profile of human macrophages, the most likely
contaminating cells, is expected to be different, because human macrophages produce neopterin on immunoactivation but do not produce substantial amounts of BH₄ at all.¹⁸

In the vascular system, BH₄ is synthesized in endothelial cells, which most likely constitute the major source of endogenous BH₄.⁸,₁²,₁⁹ Because platelets are capable of endocytosing plasma components,²⁰ it is possible that some of the BH₄ found in platelets may be of endothelial origin. However, we show in the present study that the intraplatelet pteridine profile differs considerably from the

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**Figure 2.** mRNA expression profile. Platelets were freshly isolated from healthy volunteers, and mRNA expression of BH₄ synthesis enzymes (GTPCH, PTPS, and SR) as well as cNOS mRNA was quantified by real-time RT-PCR using Light Cycler. Left, Amplification curve (cycle numbers) analysis for quantification; right, melting curve analysis for identification of specific amplicons. Each amplicon is characterized by its specific melting temperature, Tm, with a Tm of 83.9°C for GTPCH, 87.4°C for PTPS, 90.6°C for SR, and 89.8°C for cNOS. Solid lines represent results obtained from platelet RNA. Dotted lines represent results obtained with HCAECs (positive control). Dashed lines were obtained with water instead of RNA (negative control). Shown is 1 representative out of 4 independent experiments.
pteridine profile in human plasma, with the most prominent difference between intraplatelet and plasma pteridine profiles being the presence of neopterin in plasma, which stems mostly from lymphocytes and macrophages. Furthermore, the absence of neopterin, ie, the absence of accumulation of side-products of the first enzymatic step of BH₄ synthesis, GTPCH (Figure 1), indirectly suggests that the activity of the second enzyme, PTPS, is sufficient for full BH₄ synthesis in platelets. This is in sharp contrast to the low BH₄ biosynthetic capacity of human monocytes, which is caused by exon skipping in PTPS¹⁸ and results in the accumulation of neopterin.
In addition to the full BH4 de novo biosynthesis pathway, as demonstrated in this report, it was reported earlier that platelets dispose of the enzyme dihydropteridine reductase, which is involved in the regeneration of BH4 during aromatic amino acid hydroxylation reactions (Figure 1). However, although a recent study suggested a role of BH4 in thrombosis in a canine model that BH4 modulates intraplatelet NO and other BH4-dependent reactions. Our findings offer new insights into platelet functions under physiological and/or pathological conditions such as adhesion, aggregation, and thrombus formation and thereby regulate thrombosis.

In conclusion, our finding of a functional de novo BH4 synthesis in human platelets establishes the independence of platelets from external sources of this cofactor for production of NO and other BH4-dependent reactions. Our findings offer new insights into platelet functions under physiological and/or pathological conditions such as adhesion, aggregation, and thrombus formation and thereby regulate thrombosis. The clinical significance of BH4 depletion on prolonged storage in platelets certainly deserves further investigation.

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References


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