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 (BH$_4$) 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 BH$_4$ synthesis.

**Methods and Results**—We quantified mRNA expression of genes involved in BH$_4$ 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 BH$_4$ synthesis, as evidenced by the presence of mRNA species and enzymatic activity of GTP cyclohydrolase I (GTPCH), 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase. Bioppterin 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 BH$_4$ synthesis. Furthermore, our results indicate the potential of external factors, eg, prolonged storage or glucocorticoid therapy, to significantly affect BH$_4$ 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: It not only regulates vessel wall tone but also affects the hemostatic-thrombotic balance, eg, by potently inhibiting platelet activation and aggregation. Under normal conditions, the major source of NO is the endothelium, which produces NO by means of a constitutive NO synthase (cNOS). 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.

Tetrahydrobiopterin (BH$_4$) is an essential cofactor and regulator of NOS activity in the vascular system. Recently, results from a canine thrombosis model suggested an important role of intraplatelet BH$_4$ for the regulation of coronary arterial thrombosis by modulating platelet-derived NO production. However, it is as yet unknown whether BH$_4$ stems from extraplatelet sources, eg, the endothelium, or whether platelets themselves dispose over a functional BH$_4$ de novo biosynthesis, a complex multistep process. Briefly, BH$_4$ synthesis starts from GTP, which is converted to the first intermediate, 7,8-dihydronicotinamide adenine dinucleotide phosphate, 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, BH$_4$ (Figure 1). In the present study, using molecular and biochemical methods, we investigated whether the BH$_4$ de novo biosynthesis pathway is functional in human platelets. To gain preliminary insights into potential influences and regulatory mechanisms, we analyzed BH$_4$ synthesis in freshly isolated platelets from healthy donors and in platelets from healthy donors after prolonged storage. In addition, we analyzed BH$_4$ synthesis in platelets from patients treated with glucocorticoids and from patients with sepsis or septic shock conditions, known to significantly affect extraplatelet BH$_4$ synthesis.

**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 54 years (range, 25 to 60 years). They did not take any medication, especially anticoagulants, steroids, or nonsteroidal antiinflammatory drugs.

To study the isolation of inflammatory stimuli on BH₄ synthesis, platelets were isolated from blood of patients fulfilling the criteria of sepsis or septic shock. 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; normal, <3 mg/L). Mean platelet count at the time of analysis was 187 000±130 000/µL. All human studies were approved by our institutional ethics committee and conducted in accordance with the Helsinki Declaration.

**Isolation of Platelets**

Blood was drawn from healthy volunteers or patients into tubes containing 10 mmol/L sodium citrate, diluted 1:1 with Gey's balanced salt solution (Sigma)/1% human serum albumin (Swiss Red Cross Blood Bank), and centrifuged for 7 minutes at 200 g at room temperature. Platelet-enriched plasma was then collected, EDTA anticoagulation was added, and the plasma was centrifuged again for 10 minutes at 500 g at room temperature. Platelet concentrates were treated as platelet-enriched plasma. Purity of the platelets was >99.5%, and the product contained <0.04% leukocytes as determined by Coulter counter analysis.

**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. 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'-TTGGTTATCTTCTTAAACAAG3'- (forward) and 5'-GTGCTGTGCAGATTTGCT3'- (reverse); for PTPS, 5'-CGGCGACCGATTGTCAGTAA3'- (forward) and 5'-GGTGGCCAGATATAAAACGCTACA3'- (reverse); for SR, 5'-GTAACATTGACCTCCATG3'- (forward) and 5'-CTTGTTTTTCCCTTCC3'- (reverse); for cNOS, 5'-AACCCGTATAGGCTCCAGA-3' (forward) and 5'-ATGCTGTGGAAGGCTTCTT3'- (reverse); and for GAPDH, 5'-GAAAGGTGGAGGTGGTGTAACAACGG3'- (forward) and 5'-TCTCTGGAATGATGCTTGTGTTGGTTTCC-3'- (reverse). Amplification was performed as described previously.13 Values of mRNA species were corrected for 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^{−10^3} AU.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 MnO₄⁻ 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 3.6×10⁷ 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 bipterin per minute, and 1 unit of SR produces 1 μmol of bipterin 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 pteridine 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 10^10 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/10^10 platelets, and for PTPS of 5.3±0.7 μU/10^10 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/10^10 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 bipterin, the final oxidation product of BH4 and dihydrobipterin, 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 10^9 platelets contained 18.3±1.5 pmol of total bipterin 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 bipterin, 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 expression regulatory 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 pteridine levels shows a distinct pattern of abundance, with relatively high levels of bipterin, 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.

In the vascular system, BH₄ is synthesized in endothelial cells, which most likely constitute the major source of endogenous BH₄. Because platelets are capable of endocytosing plasma components, 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.\textsuperscript{21} Furthermore, the absence of neopterin, ie, the absence of accumulation of side-products of the first enzymatic step of BH\textsubscript{4} synthesis, GTPCH (Figure 1), indirectly suggests that the activity of the second enzyme, PTPS, is sufficient for full BH\textsubscript{4} synthesis in platelets. This is in sharp contrast to the low BH\textsubscript{4} biosynthetic capacity of human monocytes, which is caused by exon skipping in PTPS\textsuperscript{18} and results in the accumulation of neopterin.

Figure 3. HPLC analysis of pteridine profiles. Typical HPLC profiles of pteridine metabolites in lysates of platelets from a healthy volunteer, from stored platelets and plasma, respectively. A, Freshly isolated platelets; B, freshly obtained plasma; and C, platelets stored for 5 days. (1) indicates peak for neopterin, (2) for biopterin, and (3) for pterin.
Intraplatelet NO and superoxide production, the exact role of at least in vascular endothelial cells.12,25 It is therefore evidence that PTPS is also regulated by inflammatory stimuli, inflammatory stimuli. 8,10 Thereby, GTPCH is generally considered to be an enzyme involved in the regeneration of BH4 during formation is known that cNOS activity and subsequently NO formation is dependent on the availability of its cofactor, BH4.8 In states of BH4 deficiency, cNOS activity results in increased production of superoxide and, subsequently, the highly toxic peroxynitrite.23,24 In line with the recognition of BH4 as an important regulatory cofactor for cNOS, it has recently been shown in a canine model that BH4 modulates intraplatelet NO and superoxide production and thereby regulates thrombosis.9 Our finding of a functional intraplatelet BH4 synthesis implies that platelets might be capable of regulating their NO production intrinsically.

BH4 biosynthesis is known to be a highly regulated process that is influenced both by proinflammatory and anti-inflammatory stimuli.8,10 Thereby, GTPCH is generally considered the rate-limiting step and thus the main target for regulatory influences.10 However, we have recently provided evidence that PTPS is also regulated by inflammatory stimuli, at least in vascular endothelial cells.12,25 It is therefore conceivable to assume that BH4 biosynthesis in platelets is prone to regulation as well. Because regulation of BH4 synthesis occurs primarily at the transcriptional level,8,10 platelets themselves, as nonnucleated fragments of megakaryocytes, are unlikely to be directly affected by such regulatory influences, because they are not able to newly synthesize mRNA. Here, we found significantly lower expression of GTPCH mRNA, but not PTPS or SR mRNA, in platelets from patients treated with glucocorticoids for at least 7 days, suggesting that GTPCH gene expression can be downregulated in megakaryocytes. Prolonged therapy with glucocorticoids would therefore be expected to impair BH4 synthesis in platelets as well as in endothelial cells, and it may be speculated that this fact could contribute to the prothrombotic state and premature atherosclerosis observed in glucocorticoid-treated patients.26

Moreover, we investigated expression of BH4 synthesis in platelets of patients suffering from sepsis or septic shock to address the question of whether platelet BH4 synthesis can be regulated under these clinical conditions. Unfortunately, rapid development of thrombocytopenia with subsequent need for platelet transfusion precluded collection of blood later than 48 hours after initial diagnosis of sepsis or septic shock. At this early time point, we were unable to detect any significant differences in the expression of GTPCH, PTPS, or SR mRNA in platelets of patients with sepsis and septic shock compared with platelets from healthy volunteers (data not shown). To address the effects of proinflammatory stimuli, megakaryocytic cell lines will be needed to further study the effect of inflammatory diseases on BH4 synthesis in megakaryocytes/platelets.

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 also indicate the potential of external factors, eg, prolonged storage or glucocorticoid therapy, to significantly affect BH4 synthesis within platelets.

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References