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Functional Tetrahydrobiopterin Synthesis in Human Platelets

Nicola Frascini, PhD; Esther B. Bachli, MD; Nenad Blau, PhD; Manuel Fischler, MD; Roland B. Walter, MD; Andreas Schaffner, MD; Gabriele Schoedon, PhD

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 system¹: It not only regulates vessel wall tone but also affects the hemostatic-thrombotic balance, eg, by potentially 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,

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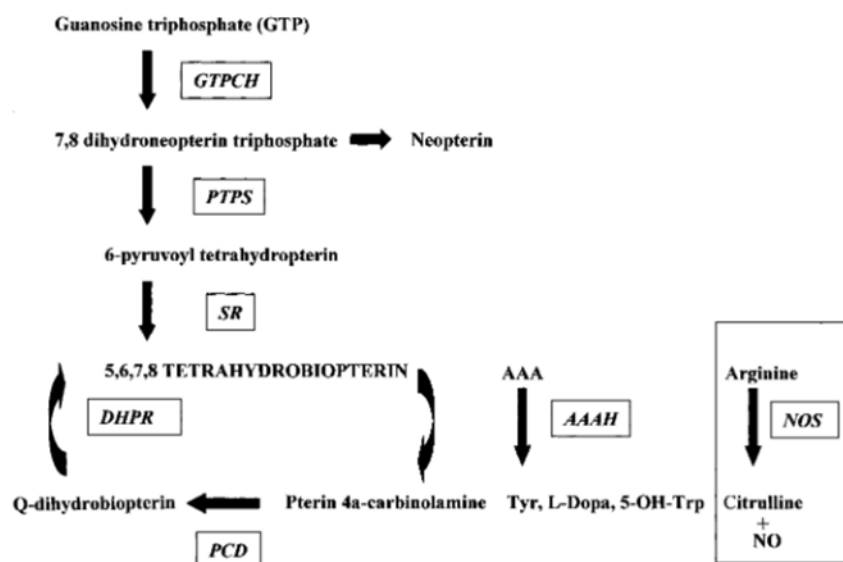


Figure 1. BH₄ de novo biosynthesis and regeneration. Biosynthesis starts from GTP, which is converted to first intermediate, 7,8-dihydroneopterin triphosphate by GTPCH. First intermediate is then converted by PTPS and subsequently by SR to end product, BH₄. Dihydropteridine reductase (DHPR) and pterin-4 α -carbinolamine reductase (PCD) are required for regeneration of BH₄ in aromatic amino acid hydroxylase reactions (AAAH); In contrast, L-arginine-NO synthesis pathway (boxed area) does not require enzymatic regeneration of BH₄. As a side-product of BH₄ synthesis pathway, first intermediate can be cleaved by phosphatases, yielding neopterin.

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, 50 to 68 years). The most frequent indication for glucocorticoid treatment was disseminated cancer with brain metastasis. Importantly, these 6 patients did not show signs of systemic inflammation, as estimated by nondetectable levels of C-reactive protein (CRP). Mean platelet count at the time of analysis was $291\,600 \pm 55\,000/\mu\text{L}$. 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; normal, <3 mg/L). Mean platelet count at the time of analysis was $187\,000 \pm 130\,000/\mu\text{L}$. 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 200g at room temperature. Platelet-enriched plasma was then collected, EDTA (final concentration: 10 mmol/L) was added, and the plasma was centrifuged again for 10 minutes at 500g 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.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'-TTGGTTATCTTCCTAACAAG-3' (forward) and 5'-GTGCTGGTCACAGTTTGTCT-3' (reverse); for PTPS, 5'-CGAGCCACCGATTGTACAGTAA-3' (forward) and 5'-GTTGTCCAGATATAAACAGCTACA-3' (reverse); for SR, 5'-CTGAACCTTGACCTCCATGC-3' (forward) and 5'-CTTGAACCTCGTCCTTTTCC-3' (reverse); for cNOS, 5'-AATCCTGTATGGCTCCGAGA-3' (forward) and 5'-ATGCTGTTGAAGCGGATCTT-3' (reverse); and for GAPDH, 5'-GGAAGGTGAAGGTCGGAGTCAACGG-3' (forward) and 5'-TCCTGGAAGATGGTGATGGGATTTC-3' (reverse). Amplicons obtained from platelet mRNA were compared with amplicons from mRNA isolated from HCAECs (positive control) 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 $E^{(35-\text{cp})}$, with E defined as PCR efficiency and determined from the slope of the standard curve as $E = 10^{-1/\text{slope}}$.^{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 MnO_2 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 3.6×10^9 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 biopterin per minute, and 1 unit of SR produces 1 μmol of biopterin per minute at 37°C.

Statistical Analysis

Results are expressed as mean \pm SEM for data from several independent experiments or mean \pm 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 10¹⁰ platelets, we measured 25.91 \pm 11.31 AU for GTPCH, 14.65 \pm 10.52 AU for PTPS, and 13.1 \pm 4.27 AU for SR (mean \pm SEM, $n=4$), respectively. Comparison with transcript levels found in HCAECs, cells with known expression levels of these genes,¹² 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 \pm 1.4 $\mu\text{U}/10^{10}$ platelets, and for PTPS of 5.3 \pm 0.7 $\mu\text{U}/10^{10}$ platelets (data are expressed as mean \pm SD of duplicate measurements from 1 representative experiment). An $\approx 10\,000$ -fold higher SR activity of 461 \pm 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 \pm 1.5 pmol of total biopterin and 10.3 \pm 1.2 pmol pterin ($n=3$) (data are mean \pm 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.^{8,10} 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 pteridine 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 with 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

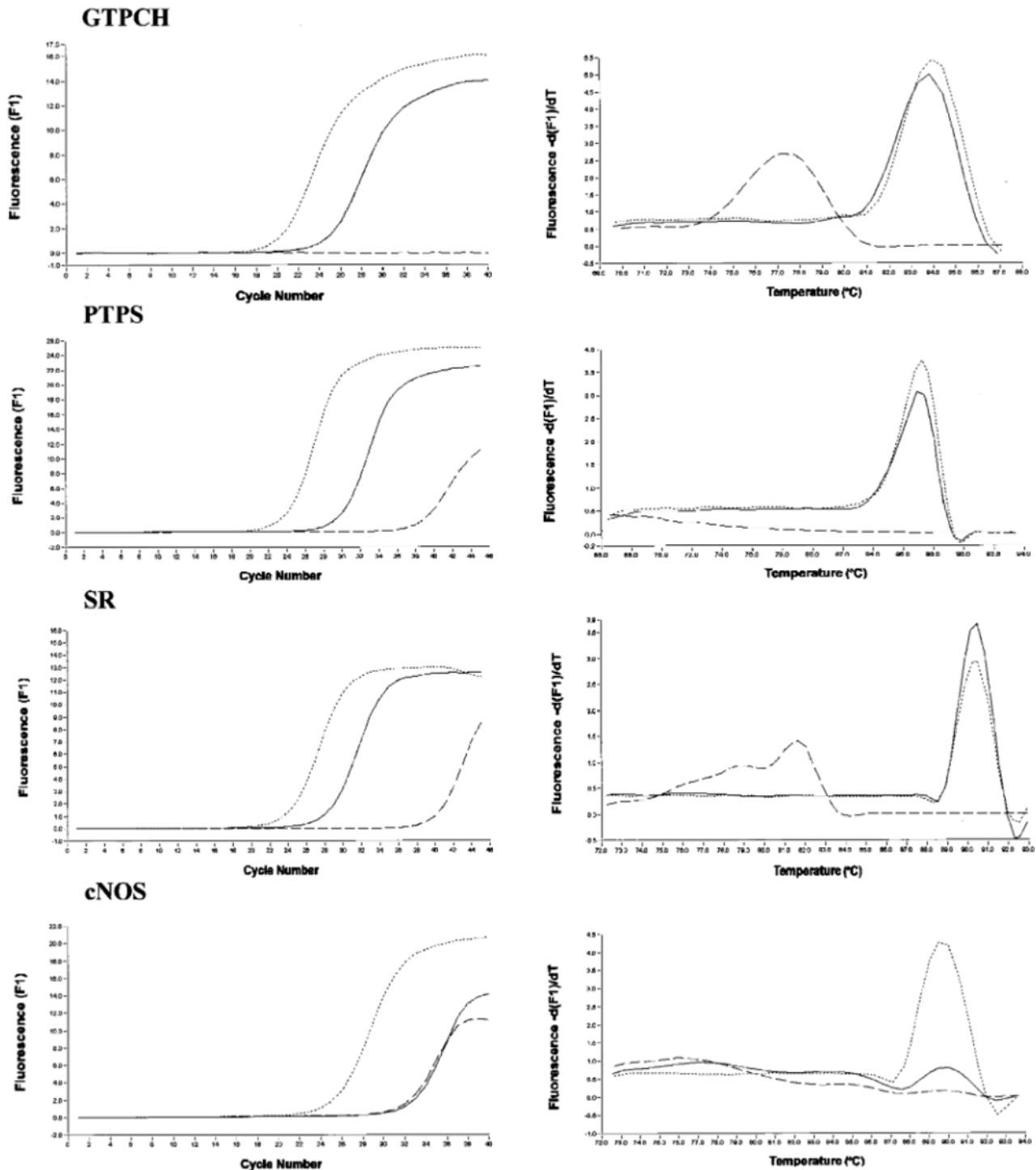


Figure 2. mRNA expression profile. Platelets were freshly isolated from healthy volunteers, and mRNA expression of BH₄ synthesis enzymes (GTPCH, PTPS, and SR) as well as cNOS mRNA was quantified by real-time RT-PCR using Light Cycler. Left, Amplification curve (cycle numbers) analysis for quantification; right, melting curve analysis for identification of specific amplicons. Each amplicon is characterized by its specific melting temperature, T_m, with a T_m 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.

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

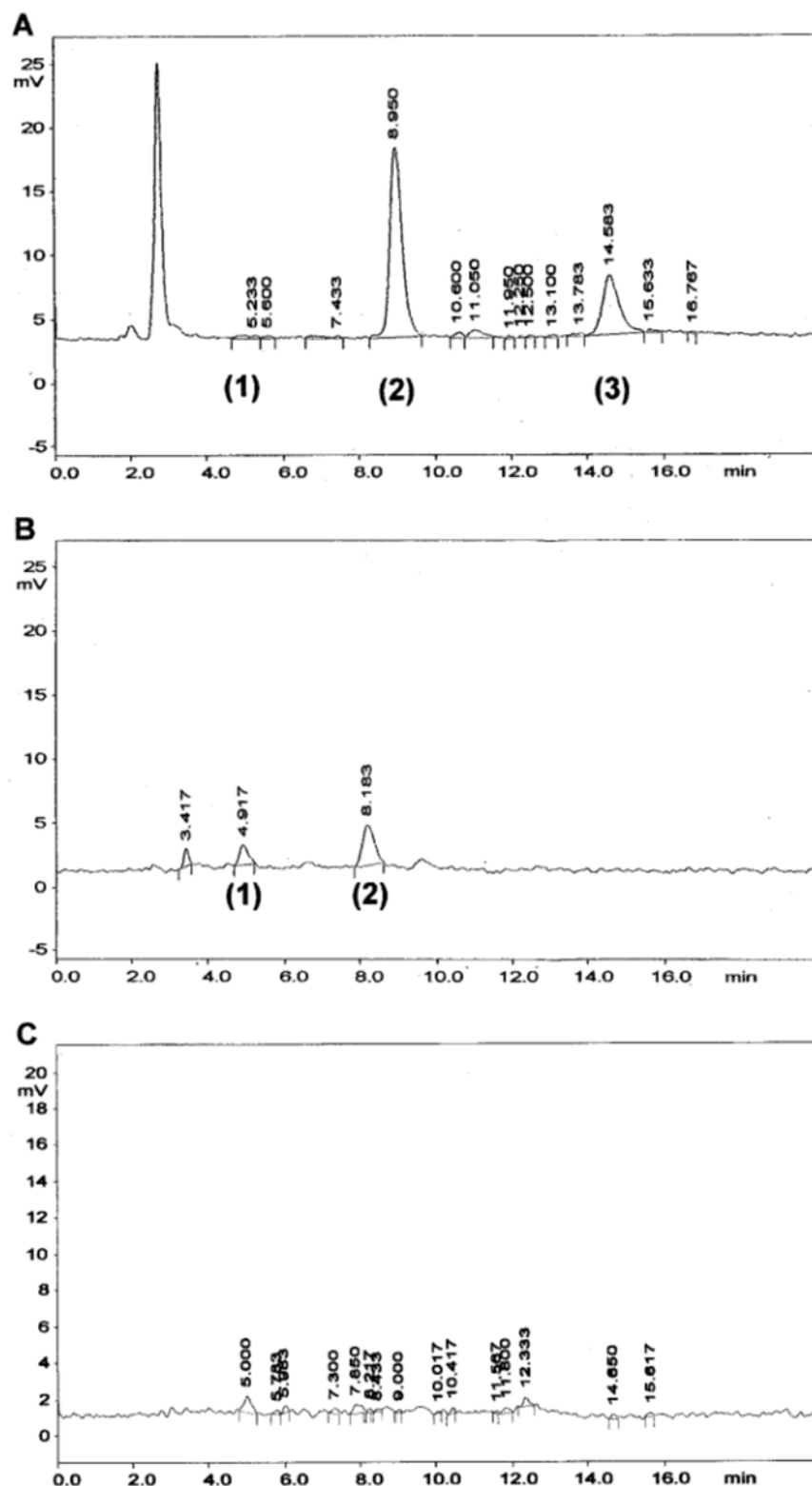


Figure 3. HPLC analysis of pteridine profiles. Typical HPLC profiles of pteridine metabolites in lysates of platelets from a healthy volunteer, from stored platelets and plasma, respectively. A, Freshly isolated platelets; B, freshly obtained plasma; and C, platelets stored for 5 days. (1) indicates peak for neopterin, (2) for biopterin, and (3) for pterin.

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.²¹ 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.

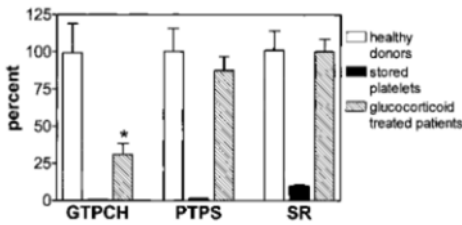


Figure 4. Influences of storage and glucocorticoid therapy on BH_4 synthesis in human platelets. Expression of mRNA species of BH_4 biosynthesis enzymes GTPCH, PTPS, and SR was calculated by normalizing means of mRNA expression from 10^{10} platelets from healthy donors to 100. Data are mean \pm SEM, $n=6$ for healthy donors and glucocorticoid treated patients; $n=4$ for stored platelets; * $P<0.0001$ (ANOVA). Open bars represent platelets from healthy volunteers, closed bars stored platelets, and hatched bars platelets from glucocorticoid-treated patients.

In addition to the full BH_4 de novo biosynthesis pathway, as demonstrated in this report, it was reported earlier that platelets dispose of functional dihydropteridine reductase,²² an enzyme involved in the regeneration of BH_4 during aromatic amino acid hydroxylation reactions (Figure 1). However, although a recent study suggested a role of intraplatelet BH_4 in thrombosis in a canine model by regulating intraplatelet NO and superoxide production, the exact role of BH_4 in platelets remains to be clarified in humans.

Several independent studies have shown that not only vascular endothelial cells⁸ but also human platelets possess a constitutive form of NOS.^{3–7} In this study, we were able to confirm the presence of cNOS mRNA expression in human platelets. Compared with endothelial cells, however, platelet cNOS mRNA expression was much less abundant. It is known that cNOS activity and subsequently NO formation is dependent on the availability of its cofactor, BH_4 .⁸ In states of BH_4 deficiency, cNOS activity results in increased production of superoxide and, subsequently, the highly toxic peroxynitrite.^{23,24} In line with the recognition of BH_4 as an important regulatory cofactor for cNOS, it has recently been shown in a canine model that BH_4 modulates intraplatelet NO and superoxide production and thereby regulates thrombosis.⁹ Our finding of a functional intraplatelet BH_4 synthesis implies that platelets might be capable of regulating their NO production intrinsically.

BH_4 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 BH_4 biosynthesis in platelets is prone to regulation as well. Because regulation of BH_4 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 BH_4 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 BH_4 synthesis in platelets of patients suffering from sepsis or septic shock to address the question of whether platelet BH_4 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 BH_4 synthesis in megakaryocytes/platelets.

In addition to the influence of clinical conditions, our data show a remarkable influence of prolonged storage on BH_4 synthesis in human platelets. Although PTPS and SR mRNA levels were significantly reduced, GTPCH expression and enzyme activity became nearly undetectable in platelets that were stored for 5 days; as a consequence, these platelets are no longer able to synthesize any pteridines. It is of note, however, that cNOS mRNA expression is still detectable at unchanged levels in stored platelets compared with freshly isolated platelets, indicating different kinetics of degradation and suggesting the potential of ongoing cNOS protein translation. The clinical significance of BH_4 depletion on prolonged storage in platelets certainly deserves further investigation.

In conclusion, our finding of a functional de novo BH_4 synthesis in human platelets establishes the independence of platelets from external sources of this cofactor for production of NO and other BH_4 -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 BH_4 synthesis within platelets.

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