Intraplatelet Tetrahydrobiopterin Plays an Important Role in Regulating Canine Coronary Arterial Thrombosis by Modulating Intraplatelet Nitric Oxide and Superoxide Generation

Seiji Kanaya, MD; Hisao Ikeda, MD, PhD; Nobuya Haramaki, MD, PhD; Toyoaki Murohara, MD, PhD; Tsutomu Imaizumi, MD, PhD

Background—Platelet-derived nitric oxide inhibits platelet aggregation via constitutive NO synthase (NOS). Tetrahydrobiopterin (BH₄), a cofactor of NOS, augments NO formation, whereas its deficiency decreases NO bioactivity and increases superoxide generation by NOS. The roles of intraplatelet BH₄ in platelet aggregation and thrombus formation, however, are unknown. Accordingly, we investigated whether intraplatelet BH₄ is involved in regulating cyclic flow variations (CFVs) and platelet aggregation in a canine model with stenosed and endothelium-injured coronary arteries that mimics acute coronary syndromes in humans.

Methods and Results—After developing CFVs, dogs received saline or BH₄ (10 or 30 mg/kg) intravenously. Intraplatelet BH₄ and cGMP levels were decreased and intraplatelet nitrotyrosine production was increased during CFVs. ADP- and U46619-induced ex vivo platelet aggregation and platelet P-selectin expression were augmented during CFVs. BH₄ administration restored intraplatelet BH₄ and cGMP levels and decreased intraplatelet nitrotyrosine production, resulting in reduced CFVs and inhibited ex vivo platelet aggregation and platelet P-selectin expression. CFVs again developed after N⁵-monomethyl-L-arginine, an inhibitor of NOS, in BH₄-treated dogs. Ex vivo platelet NOS activity at baseline, during CFVs, and after BH₄ administration did not differ.

Conclusions—Intraplatelet BH₄ may play an important role in regulating thrombus formation by modulating platelet-derived nitric oxide and superoxide generation by platelet NOS. (Circulation. 2001;104:2478-2484.)

Key Words: platelets ■ nitric oxide ■ thrombosis

Coronary arterial thrombosis has been implicated as a major pathogenic mechanism for the acute coronary syndromes.¹ In clinical studies, coronary angioscopic observations showed the presence of platelet thrombi in these syndromes.² Experimental studies have demonstrated that coronary thrombus formation produces pathophysiological manifestations similar to these syndromes.³

Platelets possess the L-arginine–nitric oxide (NO) pathway via constitutive NO synthase (NOS).⁴⁻⁵ Indeed, platelet aggregation is inhibited by L-arginine, a precursor of NO, and potentiated by N⁵-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS.⁴⁻⁶ Platelet aggregation is accompanied by an increase in the intracellular level of cGMP.⁴⁻⁶ Thus, platelet-derived NO (PDNO) production during platelet aggregation is now recognized as a negative-feedback mechanism to inhibit not only platelet aggregation but also platelet recruitment.⁸ It was recently shown that impaired PDNO production may contribute to the pathophysiology of the acute coronary syndrome.⁹

During the formation of NO from L-arginine, tetrahydrobiopterin (BH₄) acts as an essential cofactor for the catalytic activity of NOS.¹⁰ Thus, BH₄ plays an active role in the augmentation of NO production.¹⁰ In contrast, the depletion of BH₄ causes uncoupling of L-arginine to NOS, which results in the increased formation of oxygen free radicals by NOS.¹⁰ Recent clinical studies have demonstrated that exogenous administration of BH₄ restores impaired endothelial NO bioactivity in the presence of coronary risk factors,¹¹,¹² suggesting the relative deficiency of BH₄. Thus, decreased availability of BH₄ may cause a shift toward decreased NO and increased superoxide generation. Such an imbalance may, in turn, contribute to the development of atherothrombosis in coronary artery diseases. Little information is available, however, on the role of the relationship between intraplatelet BH₄ and PDNO production in platelet-mediated thrombus formation in vivo. Therefore, we hypothesized that decreased PDNO production and increased superoxide generation during thrombus formation could be related to a...
relative deficiency of intraplatelet BH₄, resulting in the further augmentation of thrombus formation in vivo. Accordingly, we examined our hypothesis by measuring intraplatelet BH₄, platelet NOS activity, PDNO production, and superoxide generation in a well-established experimental canine model of cyclic flow variations (CFVs). We also examined whether exogenous BH₄ affects intraplatelet levels of BH₄, cGMP, nitrotyrosine, and platelet NOS activity and whether exogenous BH₄ inhibits ex vivo platelet aggregation and platelet P-selectin expression.

**Methods**

**Surgical Preparation and Experimental Protocol**

All protocols were approved by the Institutional Animal Research Committee. We used the Folts coronary thrombosis model of CFVs. In this study, CFVs developed in 35 of 46 dogs. Dogs (n = 35) were divided into 4 groups. After 60 minutes of stabilization, platelets do not aggregate in response to U46619 alone, epinephrine 500 ng/mL, Cayman Co), a thromboxane mimetic. Because canine blood samples were drawn before and 2 hours after surgery (n = 11) received a bolus of BH₄ 30 mg/kg. These groups were monitored for an additional 60 minutes after treatment. The effects of each treatment on the severity of CFVs was evaluated by their frequency over a period of 60 minutes (cycles/60 minutes) and coronary blood flow velocity (CBFV) (% control). For the subsequent protocols, blood samples were drawn from the right atrium at baseline (before CFVs), during CFVs, and after treatment. Furthermore, to examine whether thoracotomy influences ex vivo platelet aggregation, intraplatelet BH₄, cGMP, and nitrotyrosine, blood samples were drawn before and 2 hours after surgery (n = 3). Consequently, thoracotomy did not influence these parameters (data not shown).

**Ex Vivo Platelet Aggregation**

To assess the effects of saline and exogenous BH₄ on platelet function in groups 1, 2, and 3, ex vivo platelet aggregation in platelet-rich plasma was studied in duplicate. Selected agonists were ADP (5 to 50 μmol/L, Sigma Chemical Co) and U46619 (50 to 500 ng/mL, Cayman Co), a thromboxane mimetic. Because canine platelets do not aggregate in response to U46619 alone, epinephrine (10 μmol/L, Sigma) was added, together with U46619. Platelet aggregation was monitored by measurement of the changes in light transmission in a platelet aggregometer (Hematracer 212, MC Medical).

**Platelet P-Selectin Expression**

To assess the platelet P-selectin expression, flow cytometric analysis was performed with FACSscan (Becton-Dickinson) as described previously.

**Intraplatelet BH₄**

How to examine intraplatelet BH₄ levels was described previously. In brief, the washed platelet suspension was homogenized with HClO₄, which immediately deproteinizes platelets, and then sonicated twice for 5 seconds with a tip sonicator and centrifuged at 12,000g for 2 minutes. BH₄ levels in the supernatant were determined by differential oxidation in acid and base with reverse-phase high-performance liquid chromatography.

**Intraplatelet cGMP**

We examined intraplatelet cGMP levels at baseline, during CFVs, and after administration of saline, BH₄, and L-NMMA. Measurements of intraplatelet cGMP levels were performed in duplicate with a radioimmunoassay kit (Yamasu Shoyu) as described previously.

**NOS Activity in Washed Platelets**

We examined ex vivo platelet NOS activity at baseline, during CFVs, and after treatment with 30 mg/kg of BH₄ in additional dogs (n = 6) of group 3. Platelet NOS activity was assessed by measuring the conversion of l-[^3]H]arginine to l-[^3]H]citrulline as described previously. For this measurement, BH₄ (3 μmol/L) was supplied as a cofactor of NOS.

**Intraplatelet Nitrotyrosine**

We examined intraplatelet nitrotyrosine production at baseline, during CFVs, and after treatment with BH₄ 30 mg/kg in additional dogs (n = 7) of group 3 using flow cytometry as described previously. For preparation of stimulated platelets, U46619 (50 ng/mL) was added to the whole blood for 5 minutes before fixation. After membrane permeabilization by methanol (−20°C, 10 minutes), immunofluorescent labeling was performed for 20 minutes with a polyclonal antibody directed against nitrotyrosine as primary antibody and FITC-conjugated goat anti-rabbit IgG antibody as secondary antibody. Furthermore, platelets obtained during CFVs were stimulated by U46619 in the presence of either 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron; 1 mmol/L), an intracellular scavenger of superoxide anion, or N⁵-nitro-L-arginine methyl ester (L-arginine methylester; L-NAME; 100 μmol/L).
CBFVs were similarly decreased among the 4 groups. The change after CFVs developed. The peak phasic and mean heart rate and systolic and diastolic aortic pressures did not among the 4 groups (Table).

**Hemodynamic Variables Before and After Treatments**

<table>
<thead>
<tr>
<th></th>
<th>Heart Rate, bpm</th>
<th>Aortic Pressure, mm Hg</th>
<th>Phasic Flow Velocity, % of Control</th>
<th>Mean Flow Velocity, % of Control</th>
<th>Frequency, CFVs/60 min</th>
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<tr>
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<td>Systolic</td>
<td>Diastolic</td>
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<td>Peak Nadir</td>
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<tr>
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<td>60±4* 8±4</td>
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<td>102±16</td>
<td>61±9* 10±9</td>
<td>70±8 11±8</td>
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</tbody>
</table>

Phasic flow velocity indicates peak phasic CBF velocity; mean flow velocity, mean CBF velocity (both phasic and mean flow are expressed as percent of unconstricted CBF velocity); peak, average high flow during CFVs; nadir, average low flow during CFVs; frequency, frequency of CFVs/60 min; baseline, time period before constrictor placement; stenosis, time period after constrictor placement and before development of CFVs; During CFVs, 60-minute observation of CFVs before treatment; and After treatment, 60-minute observation after treatment. Values are mean±SD.

*P<0.05 vs baseline; †P<0.05 vs during CFVs.

NAME, 300 μmol/L), an inhibitor of NOS. The results were expressed as percent positive nitrotyrosine staining.

**Statistical Analysis**

Values are presented as mean±SD. Statistical comparisons between groups were performed with a paired Student’s t test. Multiple comparisons were analyzed by repeated-measures ANOVA. Differences were considered significant at a value of *P<0.05.*

**Results**

**Hemodynamics**

*Before Development of CFVs*

Endothelial injury and coronary constriction decreased the averaged peak phasic CBFV to 36% to 39% of baseline and the mean CBFV to 40% to 42% of baseline. Heart rate, aortic pressure, and peak phasic and mean CBFV were comparable among the 4 groups (Table).

*During CFVs*

Heart rate and systolic and diastolic aortic pressures did not change after CFVs developed. The peak phasic and mean CBFVs were similarly decreased among the 4 groups. The phasic CBFV was decreased to 8% to 9% of baseline and the mean nadir CBFV to 9% to 10% of baseline. The averaged frequency of CFVs was 6.6 to 6.9 cycles/60 minutes. These values were similar among the 4 groups, indicating that the severity of CFVs before the treatment was similar among the 4 groups (Table).

**Effects of Treatments on CFVs**

Representative tracings are shown in Figure 1. There were no significant effects of treatments on heart rate and aortic pressure in the 4 groups (Table). Saline and BH4 10 mg/kg affected neither the nadir CBFV nor the frequency of CFVs (Table). BH4 30 mg/kg significantly increased the nadir CBFV and significantly decreased the frequency of CFVs. Thus, BH4 dose-dependently inhibited CFVs. CFVs developed again after L-NMMA in BH4-treated dogs.

**Ex Vivo Platelet Aggregation**

Ex vivo platelet aggregations to ADP and U46619 during CFVs were greater than those at baseline (Figure 2). Agonist-induced aggregations of platelets obtained during CFVs were not affected by saline. The degree of aggregations was slightly but not significantly affected by BH4 10 mg/kg. Agonist-induced aggregations were significantly suppressed by BH4 30 mg/kg, and the degree of aggregations was comparable to that at baseline.
Intraplatelet BH₄ levels during CFVs were significantly decreased compared with those at baseline in all 3 groups (Figure 3). Saline did not affect intraplatelet BH₄ levels. After treatment with 10 mg/kg of BH₄, intraplatelet BH₄ levels were significantly increased, but these levels did not differ from those at baseline. Intraplatelet BH₄ levels were significantly increased after treatment with 30 mg/kg of BH₄.

Platelet P-Selectin Expression
Platelet P-selectin expression was significantly increased during CFVs in all 3 groups (Figure 4). Saline and BH₄ 10 mg/kg did not affect P-selectin expression. BH₄ 30 mg/kg significantly decreased P-selectin expression to levels similar to those at baseline.

Intraplatelet cGMP
Intraplatelet cGMP levels were significantly decreased during CFVs in the 4 groups (Figure 5). Saline did not affect intraplatelet cGMP levels. BH₄ 10 mg/kg increased intraplatelet cGMP levels, but these levels were still lower than those at baseline. BH₄ 30 mg/kg increased intraplatelet cGMP to similar levels at baseline. The restored intraplatelet cGMP levels after treatment with BH₄ 30 mg/kg were decreased again after L-NMMA.

Platelet NOS Activity
Ex vivo platelet NOS activity at baseline, during CFVs, and after treatment with 30 mg/kg BH₄ was 1.02±0.1, 1.02±0.1, and 1.03±0.1 pmol l-citrulline/min per 10⁸ platelets, respectively (P=NS).

Intraplatelet Nitrotyrosine Production
The left row of Figure 6 shows representative histograms of intraplatelet nitrotyrosine production. The intraplatelet nitrotyrosine production during CFVs was significantly greater than that at baseline. After treatment with BH₄ 30 mg/kg, the production was significantly decreased to levels similar to those at baseline. The increase in intraplatelet nitrotyrosine production during CFVs was not observed when nonspecific IgG was used instead of the nitrotyrosine antibody or when the nitrotyrosine antibody was applied in the presence of excess (10 mmol/L) soluble nitrotyrosine (data not shown). Intraplatelet nitrotyrosine production during CFVs in the presence of Tiron (1.35±0.3%) or L-NAME (1.19±0.4%) was similar to the levels observed at baseline, indicating that nitrotyrosine is a “footprint” of peroxynitrite.

Discussion
The major findings of the present study are that (1) there were decreases in intraplatelet levels of BH₄ and cGMP and increases in intraplatelet nitrotyrosine production during CFVs; (2) exogenously administered BH₄ dose-dependently restored intraplatelet levels of BH₄ and cGMP and decreased intraplatelet nitrotyrosine production, resulting in reduced CFVs and in inhibited ex vivo platelet aggregation and platelet P-selectin expression; (3) L-NMMA administered exogenously after elimination of CFVs by BH₄ administration decreased intraplatelet cGMP levels, and CFVs again developed; and (4) platelet NOS activity at baseline, during CFVs, and after treatment with BH₄ did not differ. Thus, the present findings demonstrate that intraplatelet BH₄ may be critically involved in the regulation of thrombus formation by modulating PDNO production and superoxide generation by platelet NOS.

To assess whether the thrombotic process during CFVs is related to a decrease in intraplatelet BH₄, we measured intraplatelet BH₄. Intraplatelet BH₄ levels during CFVs were decreased compared with those at baseline. Expression of platelet P-selectin, an important molecule for the platelet-leukocyte adhesion, during CFVs was increased compared with those at baseline. This finding is consistent with the results of our recent study. Taken together, decreased intraplatelet BH₄ during thrombus formation might enhance platelet aggregation and adhesiveness, resulting in augmented thrombus formation.

On the basis of these findings, we considered that intracellular BH₄ may be a major regulator for the thrombotic process.
during CFVs. We therefore examined the effects of BH₄ treatment on CFVs in the present model. When BH₄ was administered exogenously during CFVs, the intraplatelet BH₄ level was increased dose-dependently. BH₄ at 30 mg/kg reduced CFVs without any systemic hemodynamic changes. Although BH₄ reduced CFVs in vivo, it was unknown whether the effects of BH₄ on CFVs were related to the inhibition of platelet aggregation. We therefore examined the effect of BH₄ on ex vivo platelet aggregation during CFVs. ADP and U46619, a thromboxane mimetic, were chosen as platelet agonists because ADP and thromboxane A₂ are important mediators of CFVs. BH₄ at 30 mg/kg suppressed platelet aggregation, and the magnitude of platelet aggregation was similar to the level at baseline. Furthermore, BH₄ at 30 mg/kg suppressed platelet P-selectin expression, and the magnitude of platelet P-selectin expression was similar to the level at baseline. Thus, our data suggest that BH₄ treatment prevented thrombus formation by inhibiting platelet aggregation and by suppressing P-selectin-mediated platelet-leukocyte interactions. The intraplatelet BH₄ level after treatment with BH₄ 10 mg/kg was comparable to that at baseline; thus, CFVs were not abolished and ex vivo platelet aggregation in response to agonists was not inhibited, nor was platelet P-selectin expression inhibited. Thus, it is probable that once CFVs have occurred, the restoration of BH₄ to the baseline level is not sufficient to eliminate CFVs. This issue is further addressed below.

To investigate the role of intraplatelet BH₄ in thrombus formation in vivo, we measured intraplatelet cGMP as a marker of PDNO production. We have previously shown that PDNO is a regulator of P-selectin expression in platelets; PDNO inhibits platelet P-selectin expression. The positive relationship between intracellular BH₄ and NO production has been demonstrated in endothelial cells. Furthermore, we and others have previously demonstrated that the intraplatelet cGMP is a second messenger of NO in platelets and that inhibition of platelet aggregation by PDNO is mediated via cGMP. In this study, intraplatelet cGMP levels were decreased during CFVs, indicating decreased intraplatelet PDNO during thrombus formation. When BH₄ 30 mg/kg was exogenously administered during CFVs, intraplatelet BH₄ and cGMP were restored. Furthermore, when L-NMMA was administered exogenously after elimination of CFVs by BH₄ at 30 mg/kg, intraplatelet cGMP levels were decreased and CFVs developed again. Thus, our data suggest that intraplatelet BH₄ is a modulator of platelet-mediated thrombus formation via PDNO production as a cofactor of platelet NOS. BH₄ at 10 mg/kg did not restore intraplatelet cGMP.
levels to those at baseline, which probably accounts for the ineffectiveness of the low-dose BH₄ for elimination of CFVs and inhibition of platelet aggregation and P-selectin expression.

To further investigate the involvement of intraplatelet BH₄ for thrombus formation, we measured intraplatelet nitrotyrosine production as a marker of peroxynitrite formation in vivo, because BH₄ has been shown to be a primary target for peroxynitrite-catalyzed oxidation in vitro.²⁶ In the BH₄-depleted state, superoxide is produced by NOS in vitro.¹⁰ The reaction between NO and superoxide can produce the powerful oxidant peroxynitrite, which forms nitrotyrosine. In this study, intraplatelet BH₄ was decreased and intraplatelet nitrotyrosine production was increased during CFVs, suggesting the occurrence of peroxynitrite-catalyzed oxidation during thrombus formation. Ex vivo platelet NOS activity during CFVs, however, did not differ from that at baseline. It should be kept in mind that, for measurement of NOS activity, a sufficient amount of exogenous BH₄ as a cofactor of NOS has to be supplied. The results indicate that platelet NOS obtained during CFVs can produce a proper level of PDNO if a sufficient amount of intraplatelet BH₄ is present. Therefore, the present findings suggest that the decreased intraplatelet BH₄ level (as a cofactor of NOS) during thrombus formation caused uncoupling of L-arginine to NOS in vivo, resulting in decreased PDNO production and increased superoxide generation by platelet NOS. The superoxide produced may have further reduced intraplatelet BH₄, resulting in a vicious circle. Thus, the local event may spread systemically. Taken together, our data indicate that decreased intraplatelet BH₄ causes an imbalance between the protective PDNO production and deleterious intraplatelet superoxide generation by NOS, resulting in the augmentation of thrombus formation.

The present study has some limitations. First, in previous studies, we and others demonstrated that oxygen free radicals are important mediators of CFVs.²⁷–²⁹ In this study, it may be considered that superoxide is generated via NOS during thrombus formation. During thrombus formation, however, superoxide is also generated by cyclooxygenase³⁰ and xanthine oxidase.²⁹ And there are several other potential pathways as sources of oxygen free radicals in the thrombotic process. Thus, intraplatelet BH₄ may have been decreased by increased superoxide generation, probably via not only NOS but also other pathways. Second, in the present study, treatment with BH₄ 30 mg/kg did not completely abolish the

**Figure 5.** Intraplatelet cGMP levels at baseline, during CFVs, after administration of BH₄, and after administration of BH₄+L-NMMA.

![Graphs showing intraplatelet cGMP levels](image)

**Figure 6.** Left, Representative histograms of intraplatelet nitrotyrosine production at baseline, during CFVs, and after treatment with BH₄ 30 mg/kg. Note that nitrotyrosine production was increased during CFVs, as indicated by rightward shift in histogram. After BH₄ treatment, nitrotyrosine production was decreased to level before CFVs.
episode of CFVs. Possible explanations may be that important mediators of CFVs are not only intraplatelet BH₄ but also thromboxane A₂, 12-hydroxyeicosatetraenoic acid, ADP, serotonin, thrombin, and oxygen free radicals. Finally, we did not examine the effect of exogenous BH₄ on endothelial dysfunction in this study. Because the endothelium at the stenotic site was mechanically disrupted, however, the effect of exogenous BH₄ has no place to act.

In conclusion, the present study, to the best of our knowledge, provides the first line of evidence that intraplatelet BH₄ plays an important role in regulating thrombus formation by modulating PDNO and superoxide generation by platelet NOS in vivo. Our data suggest that decreased intraplatelet BH₄ may contribute to the pathophysiology of thrombus formation in acute coronary syndromes. Because NOS-mediated superoxide generation is decreased by exogenous BH₄, BH₄ supplementation may become a novel therapeutic approach to these syndromes in humans.

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References
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