Deficient Platelet-Derived Nitric Oxide and Enhanced Hemostasis in Mice Lacking the NOSIII Gene

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Abstract—Endothelial nitric oxide synthase (eNOS) has been identified in human platelets. Although platelet-derived nitric oxide (NO) has been shown to inhibit platelet recruitment in vitro, its role in the regulation of the hemostatic response in vivo has not been characterized. To define the role of platelet-derived NO in vivo, we studied mice that lacked a functional eNOS gene (NOSIII). Surface P-selectin expression in platelets from eNOS-deficient mice was not significantly altered; however, bleeding times were markedly decreased in eNOS-deficient versus wild-type mice (77.2±3 versus 133.4±3 seconds, \( P<0.00005 \)). To determine the contribution of endothelium- versus platelet-derived NO to the bleeding time, isolated platelets from either eNOS-deficient or wild-type mice were transfused into a thrombocytopenic eNOS-deficient mouse and the bleeding time was measured. The bleeding times in mice transfused with eNOS-deficient platelets were significantly decreased compared with mice transfused with wild-type platelets (Δbleeding time, \(-24.6±9.1\) and \(-3.4±5.3\) seconds, respectively; \( P<0.04 \)). Platelet recruitment was studied by measuring serotonin release from a second recruitable population of platelets that were added to stimulated platelets at the peak of NO production. There was 40.3±3.7% and 52.0±2.1% serotonin release for platelets added to wild-type or eNOS-deficient platelets, respectively (\( P<0.05 \)). In summary, mice that lacked eNOS had markedly decreased bleeding times even after endothelial NO production was controlled. These data suggest that the lack of platelet-derived NO alters in vivo hemostatic response by increasing platelet recruitment. Thus, these data support a role for platelet-derived NO production in the regulation of hemostasis. (Circ Res. 1999;84:1416-1421.)

Key Words: selectin • mice • platelet • nitric oxide synthase

Normal hemostatic balance is maintained by tight regulation of platelet activation and recruitment. Adhesion of platelets to the endothelium is prevented by several mechanisms, including endothelial cell production of nitric oxide (NO).1-3 NO inhibits platelet adhesion and aggregation4-14 and prevents thrombosis.3 Exogenous NO inhibits the normal activation-dependent increase in the expression of platelet surface glycoproteins, including P-selectin and the integrin glycoprotein IIb/IIIa complex.4 In addition, we have shown that a functional decrease in exogenous NO can lead to a clinical thrombotic disorder.5,6

In addition to its presence in endothelial cells, constitutive NO synthase (NOS) has been identified in human platelets and megakaryocytic cells.9-12 Consistent with these observations, studies report NO release from aggregating platelets.13-15 Platelet aggregation is enhanced by incubation with inhibitors of NOS and inhibited by incubation with the NOS substrate L-arginine.16 Although platelet-derived NO appears to inhibit the primary aggregation response only modestly, we have recently shown that NO release from activated human platelets markedly inhibits platelet recruitment15 and thus may attenuate the progression of intra-arterial thrombosis. In vivo, systemic infusion of a NOS inhibitor causes a reduction in bleeding time without changing vessel tone17 and enhances platelet reactivity to various agonists,18 which supports the clinical relevance of platelet-derived NO. In addition, it is well established that thrombosis is the usual cause of unstable angina and myocardial infarction19,20 and, more importantly, activated platelets from patients with these acute coronary syndromes produce significantly less NO versus patients with stable coronary artery disease.21 This observation suggests that impaired platelet-derived NO may contribute to the development of acute coronary syndromes by enhancing platelet function or recruitment and subsequently thrombus formation.

Homozygous eNOS-mutant mice have been studied and are known to have impaired endothelium-derived relaxing factor activity,22 increased blood pressure, decreased heart rate, and increased plasma renin concentration.23 In the pulmonary vasculature, eNOS deficiency produces mild pul-
monary hypertension. Although vascular reactivity has been extensively characterized in these mice, hemostatic and thrombotic responses have not yet been studied. Therefore, eNOS-mutant mice were examined to define the role of platelet-derived NO in platelet function and hemostasis.

Materials and Methods

All studies were approved by the Institutional Animal Care and Use Committee at Boston University. The generation of mice with the NOSIII gene deletion has been previously described in detail. Ten-week-old male mice that weighed 22 to 24 g were used for all experiments. Mice deficient in eNOS- were compared with both SV-129 and C57 wild-type mice (Charles River Laboratories, Cambridge, Mass), which represented the strains from which the eNOS-deficient mice (Massachusetts General Hospital) were derived. Blood was drawn into syringes and anticoagulated with trisodium citrate by inferior vena cava puncture. Bone marrow cells were isolated from euthanized mice by gently perfusing the marrow cavity of isolated femurs with 10 mmol/L sodium phosphate, pH 7.2, and 150 mmol/L NaCl after extirpation of the proximal and distal metaphyses.

Reverse Transcription–Polymerase Chain Reaction for Mouse NOSIII and P-Selectin

Total RNA was extracted from bone marrow cells with a commercial solution that contained guanidinium thiocyanate (RNASol, Cinna/Biotex). Contaminating DNA was digested with RNase-free DNaseI (Boehringer-Mannheim Biochemicals). Reverse transcription (RT) was performed with 150 ng of total RNA in 20 μL of a reaction mixture that contained 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L of each dNTP, 200 U of superscript reverse transcriptase (Gibco BRL), and 10 μg/mL oligo-dT 16. After incubation at 42°C for 1 hour, the reaction was stopped by incubation at 70°C for 5 minutes. A nested primer polymerase chain reaction (PCR) was performed with 10 μL of the reaction mixture from the RT reaction in 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 200 μmol/L of each dNTP, and 2.5 U Taq DNA polymerase (Boehringer-Mannheim). Primer sequences used for the first and second PCR reactions for eNOS were 5′-gtagttctactctgtcagcac and 5′-tattttggtcgcgctgggtctaa, and 5′-gagttcctgcttccccttcata and 5′-tggctccctggtggctgaat, respectively. Primer sequences used for the first and second PCR reactions for P-selectin were 5′-tccagaggaaactgtcaggctg and 5′-tggtgtctgcatgtactcgagag, and 5′-gtcctacaggtccttgtcatctgcggctgttcttg and 5′-ggtttcagcaggctggctgtcttctgcggctgttcttg, respectively. Primers were added at a final concentration of 1.0 mmol/L. Amplification was performed in a DNA thermal cycler. A second PCR reaction was performed with 10 μL of the original PCR mixture and the nested primer pairs under the same conditions. Amplified products were analyzed by 1.5% agarose gel electrophoresis and visualized under UV illumination after they were stained with ethidium bromide.

Preparation of Platelets

The blood was centrifuged (150g, 10 minutes, 22°C) and the supernatant, which represented platelet-rich plasma (PRP), was separated. Gel-filtered platelets (GFP) were prepared by passing PRP over a Sepharose-2B column equilibrated with Tyrode’s-HEPES buffered saline as previously described. Platelet counts were determined with a Coulter Counter, model ZM (Coulter Electronics).

Measurement of Platelet NO Production and Aggregation

We adapted a NO-selective26 microelectrode (Inter Medical Co Ltd) for use in a standard platelet aggregometer (Payton Associates) to monitor platelet NO production and aggregation simultaneously as previously described. Platelet NO production was quantified as the integrated signal detected by the microelectrode after platelet acti-

vation with 5 μmol/L ADP. Aggregation of GFPs was monitored with a standard nephelometric technique as previously described.27,28

Measurement of Platelet Surface P-Selectin Expression by Flow Cytometry

Resting or epinephrine-activated (100 μmol/L) platelets in whole blood were incubated with CD62 (rabbit anti–P-selectin antibody) in PBS for 15 minutes at 22°C. This concentration of epinephrine causes the maximal aggregation response in mouse platelets. Samples were then fixed in paraformaldehyde (1% FC) and incubated for 30 minutes at 22°C. Samples were diluted 10-fold in PBS, and the cells were collected by centrifugation (1700g, 5 minutes). The samples were incubated with PE-GaR (BioSource International) for 15 minutes at 22°C. After the samples were washed with PBS, they were incubated with FITC-D9 (CD41 rat anti-mouse antibody) and incubated for 15 minutes at 22°C. Samples were resuspended in PBS and analyzed in a flow cytometer (FACScan, Becton Dickinson) with settings for FL1 fluorescence to measure the FITC D9-labeled platelets. Platelets were gated with a forward scatter versus FL1 dot plot and a gated histogram of forward scatter versus FL2 to separate activated from unactivated platelets.

Measurement of Bleeding Time

Bleeding times were measured by determining the time required for clotting to occur after a single puncture (2 mm) with a 23-gauge needle to the dorsal tail vein. The bleeding times were conducted in duplicate in each animal. During the bleeding times, body temperature was maintained at 36°C to 38°C by placing the mice on heating pads. Before the mice were killed, blood samples were drawn from them for measurement of platelet-derived NO and aggregation studies.

For some experiments, platelets were isolated, pooled, and infused into eNOS-deficient mice that were rendered thrombocytopenic with a single intraperitoneal injection of carboplatin (Bristol-Myers Squibb), a chemotherapeutic agent that causes thrombocytopenia at a nonlethal dose.29 Carboplatin was injected intraperitoneally at a dose of 125 mg/kg to induce thrombocytopenia. By use of this method, platelet counts in mice decreased by >80% ~12 days after injection. Platelets were infused 12 days after injection, after confirmation of decreased platelet count. Approximately 1.2×1010 platelets were reinfused into each animal. Bleeding times were determined before and after the platelet infusion.

Platelet Secretion

Platelet secretion was measured with [14C]–radionabeled serotonin as previously described.30,31 GFPs were incubated with [14C] serotonin at 37°C for 10 minutes. Imipramine was added immediately before the initiation of secretion to prevent reuptake of secreted serotonin. Secretion was initiated by the addition of 10 μmol/L ADP and allowed to proceed for 2 minutes and then terminated by the addition of ice-cold formaldehyde in 0.05 mol/L EDTA. Samples were spun in a microfuge at 14 000g for 3 minutes, and radioactivity was measured in the supernatant.

Thromboxane B2 in Platelets

Platelet isolates (resting and epinephrine-stimulated) from NO-deficient or control mice were precipitated with 10% trichloroacetic acid.32 After centrifugation and extraction, thromboxane B2 levels were measured by enzyme immunoassay (Cayman Chemicals). The results were expressed as nanograms of thromboxane B2 per 1×109 platelets.

Statistical Analysis

Differences between groups were determined with an unpaired Student t test. The effects of the interventions were analyzed by a paired t test. A statistically significant difference was assumed with a value of P<0.05. All data are expressed as the mean±SEM.
Results

Absence of eNOS RNA in Bone Marrow

Previous genetic characterization of eNOS-deficient mice has not examined megakaryocytic cell lines. Therefore, to confirm the absence of eNOS expression in these cells, RNA was extracted from the bone marrow samples of eNOS-deficient and wild-type (c57) animals and amplified by RT-PCR. As shown in Figure 1, eNOS-deficient animals do not express NOSIII mRNA in marrow cells (lane 1), although wild-type animals do (lane 2). This result contrasts with the expression of P-selectin, which is present in both groups of mice.

Platelet NO Production

To determine whether the lack of eNOS alters aggregation-dependent NO production in the mouse platelet, NO production was measured with a selective electrochemical detector adapted for use in a platelet aggregometer. GFPs were stimulated with 5 μmol/L ADP, and aggregation and NO release was determined. As can be seen in the representative tracing (Figure 2A), the eNOS-deficient platelets do not release measurable NO compared with the stimulated platelets from both c57 (Figure 2A) and SV-129 (data not shown) wild-type animals. The consistency of this finding is demonstrated in the cumulative results shown in Figure 2B. Comparable levels of platelet NO release were detected with the use of thrombin and collagen as platelet agonists (data not shown).

Platelet Surface P-Selectin Expression in Resting and Stimulated Platelets

Because mice yield insufficient quantities of blood to perform complete studies of platelet function with standard aggregometry, the extent of platelet activation was assessed by flow cytometry. Both resting and stimulated platelets were assessed for surface expression of P-selectin, a marker of platelet activation, by use of a rabbit antibody specific for mouse P-selectin. As is shown in Figure 3, the normal stimulation-dependent increase in platelet surface expression of P-selectin is present in both eNOS-deficient and wild-type mice. Interestingly, there is no significant difference in either of these levels between the eNOS-deficient and wild-type animals.

Bleeding Time Measurements in eNOS-Deficient and Wild-Type Mice

To determine whether the absence of platelet NO release results in enhanced hemostasis, bleeding times in eNOS-deficient (NOSIII$^{-/-}$) and wild-type (NOSIII$^{+/+}$) mice were measured. Compared with the wild-type mice (c57), bleeding times from eNOS-deficient animals were significantly decreased (bleeding time, 124.6±3 versus 77.2±3 seconds, $P<0.00005$, Figure 4). This finding was confirmed by use of SV-129 control mice (126±4 versus 62.6±3 for SV-129...
versus eNOS-deficient mice, respectively; $P<0.00005$, $n=5$ for both).

The integrity of hemostasis, which was measured by the bleeding time, is influenced both by endothelial and platelet function. To determine the contribution of platelet- versus endothelium-derived NO on the bleeding time and in vivo hemostasis, platelets from eNOS-deficient or wild-type animals were transfused into thrombocytopenic eNOS-deficient mice. Bleeding times were measured before and after the transfusion, and the change in bleeding time was determined. Bleeding times were decreased in most animals after platelet infusion. More importantly, mice transfused with wild-type platelets had less of a decrease in bleeding times versus those transfused with eNOS-deficient platelets ($\Delta$bleeding time, $-24.6 \pm 9.1$ versus $-3.4 \pm 5.3$ seconds; $P<0.04$, Figure 5).

**Effect of eNOS Deficiency on Platelet Recruitment**

Although the prolonged bleeding times (Figure 5) suggest that platelet NO production contributes to hemostasis, no abnormality was detected in the platelet activation response in eNOS-deficient animals (Figure 3). With an inhibitor of NOS, we have previously shown that decreasing platelet-derived NO modestly attenuates platelet activation but markedly inhibits platelet recruitment. Therefore, to determine whether the hemostatic changes detected in the eNOS-deficient animals are due to alterations in platelet recruitment, we used $[^{14}C]$serotonin release, a measure of platelet-dense granule secretion, as an index of platelet activation.15 GFPs from wild-type or eNOS-deficient animals were stimulated with ADP and, at the peak of NO release, $[^{14}C]$serotonin-loaded wild-type platelets were added and platelet secretion was determined by measuring the release of $[^{14}C]$serotonin ($P<0.05$, $n=5$).

Platelets were added to wild-type or eNOS-deficient platelets, there was $44.3 \pm 2.9\%$ and $52.0 \pm 2.1\%$ serotonin release, respectively (Figure 6; $P<0.05$).

To confirm that the change in serotonin release was due to the absence of NO, this experiment was repeated with platelets from wild-type animals that had been incubated with the NOS inhibitor $N^\omega$-nitro-$L$-arginine methyl ester (L-NAME). GFPs treated with 300 $\mu$mol/L of L-NAME or vehicle control were stimulated with ADP and, at the peak of NO production, unstimulated $[^{14}C]$serotonin-loaded control GFPs were added and serotonin release was measured. When $[^{14}C]$serotonin-containing platelets were added to control or NO-inhibited platelets, there was a $35 \pm 1.8\%$ or $47.3 \pm 3.1\%$ serotonin release, respectively ($P<0.05$, $n=3$).

**Effect of eNOS Deficiency on Thromboxane Activity**

Although bleeding time measurements determined the contribution of platelet- versus endothelium-dependent NO release to hemostasis, these studies did not explore other hemostatic mechanisms that may be influenced by the presence or absence of NO. Previously, platelet thromboxane synthase activity was shown to be attenuated by NO.33 Therefore, the contribution of endogenous NO to platelet thromboxane activity was determined by measuring thromboxane B$_2$, the chemically stable hydration product of thromboxane A$_2$. There was no difference in thromboxane B$_2$ formation in activated platelets from control or NO-deficient mice ($0.55 \pm 0.13$ versus $0.61 \pm 0.16$ ng per $10^8$ platelets, respectively; $n=4$ experiments, $P=ns$).

**Discussion**

NO has been previously shown to be a potent inhibitor of platelet function. Although endothelium-derived NO production is known to both mediate vasorelaxation as well as inhibit platelet adhesion and activation, less is known about the contribution of platelet-derived NO. In this study, which used mice that lacked the NOSIII gene, we demonstrate that platelet-derived NO production contributes to hemostasis in vivo. After confirming the lack of NOSIII expression in the marrow (megakaryocytes) of these animals, stimulated platelets were found to lack stimulation-dependent NO release.
These animals were also found to have significantly shortened bleeding times. Also, after controlling for endothelium-dependent NO production by inducing thrombocytopenia and transferring wild-type or eNOS-deficient platelets into these animals, they still had reduced bleeding times, which suggests that NO release by both the endothelium and the platelet contributes to hemostasis.

Although the platelet reinfusion experiment determined the contribution of platelet- versus endothelium-dependent NO release to the bleeding time, it did not control for other hemostatic mechanisms that may be influenced by the presence or absence of NO. Previously, platelet thromboxane synthase activity was shown to be attenuated by NO. In our study, there was no significant difference in levels of thromboxane B2 in eNOS-deficient versus control mice. However, previous studies that detected NO-dependent changes in thromboxane activity used exogenous NO donors at concentrations that greatly exceed levels of endogenous NO release.

As discussed, platelets normally adhere to the subendothelium after intimal injury, which leads to platelet activation. Once activated, platelets promote thrombus growth and recruit additional platelets to the growing thrombus by the release of ADP and serotonin, production of thromboxane A2, and promotion of surface thrombin generation. This process is known as the recruitment phase of platelet activation. Interestingly, lack of NO production by platelets was not associated with alterations in stimulation-dependent surface P-selectin expression. However, deficiency in platelet-NO production was associated with enhanced platelet activation in a “recruitable” platelet population assessed by serotonin release. This is consistent with our previous characterization of NO production by the human platelet.

In this study, the alteration of NO production in platelets that used NOS inhibitors minimally altered platelet activation. By use of a double-labeling technique, platelet-derived NO markedly inhibited recruitable platelets that were added during the peak of NO release. This study suggested that platelet-derived NO regulated platelet recruitment; however, it did not address the in vivo contribution of platelet-derived NO release.

Although platelet NO release does not have a major effect on the primary aggregation response, platelet-derived NO appears to play an important counterregulatory role after platelet activation by inhibiting the recruitment of platelets to the growing thrombus. Because platelet recruitment is a critical component in thrombus propagation, it is reasonable to speculate that platelet-derived NO release may play a role in the regulation of hemostasis. In addition, we have recently shown that aggregating platelets from patients with acute coronary syndromes produce less NO. Because platelet aggregation and thrombus formation are implicated in unstable angina and myocardial infarction, impaired platelet-derived NO production may also contribute to the development of acute thrombotic events.

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