Rapid Regulation of Platelet Activation In Vivo by Nitric Oxide

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Background—Platelet activation is a feature of many cardiovascular diseases characterized by endothelial dysfunction. The mechanistic relationship between impaired systemic nitric oxide (NO) bioavailability and platelet activation in vivo remains unclear. We investigated whether acute inhibition of NO production in humans modulates platelet activation in vivo and whether exogenous NO would counteract such an effect.

Methods and Results—Intravenous injection of the NO synthase inhibitor Nω-monomethyl-L-arginine in healthy volunteers resulted in NO synthase inhibition as detected by increased blood pressure and by significantly reduced phosphorylation of platelet vasodilator-stimulated phosphoprotein, an indicator of NO signaling. NO synthase inhibition increased platelet activation as determined by enhanced platelet binding of fibrinogen and surface expression of P-selectin, glycoprotein 53, and CD40 ligand, demonstrating tonic inhibition of platelet activation by NO production in vivo. Sublingual administration of the NO donor glyceryl trinitrate normalized platelet VASP phosphorylation and restored markers of platelet activation to baseline levels.

Conclusions—Acute inhibition of endogenous NO production in humans causes rapid platelet activation in vivo, which is reversed by exogenous NO, demonstrating that platelet function in vivo is rapidly regulated by NO bioavailability. (Circulation. 2004;109:1819-1822.)

Key Words: glycoproteins □ nitric oxide □ P-selectin □ phosphoproteins □ platelets

Nitric oxide (NO), generated by NO synthase (NOS), inhibits platelet activation1 as demonstrated by in vitro experiments such as inhibition of platelet aggregation by endothelial cells2 or NO donors.3,4 NO can even reverse agonist-induced activation of glycoprotein IIb/IIIa.5 Chronic inhibition of NO formation in animal models is associated with impaired fibrinolysis and enhanced thrombin and tissue factor generation.6 Previous reports described increased platelet activation in disease states with impaired NO bioavailability such as acute coronary syndromes,7 heart failure,8 diabetes,9 and hypercholesterolemia.10 However, the direct relationship between NO bioavailability and platelet activation in humans remains unclear.

NO-dependent phosphorylation of vasodilator-stimulated phosphoprotein (VASP) is an essential regulatory component in the inhibition of platelet activation and correlates with inhibition of fibrinogen binding to glycoprotein IIb/IIIa.11,12 P-selectin expression, and platelet adhesion.13 With specific antibodies, VASP phosphorylation provides a sensitive monitor of defective NO/cGMP signaling, and reduced NO bioavailability in several pathophysiological states correlates with reduced VASP phosphorylation.14

In the present study, we investigated whether acute changes in NO bioavailability in healthy human volunteers would affect platelet activation as determined by activity of the fibrinogen receptor and platelet degranulation.

Methods
Venous blood was taken from 12 healthy nonsmoking male donors (age, 29±1.3 years) resting in a supine position and was collected into a tube prepared with sodium citrate (3.8%). Blood samples were collected under baseline conditions, 30 minutes after intravenous injection with Nω-monomethyl-L-arginine (L-NMMA; Calbiochem-Novabiochem; 5 mg/kg given as a bolus over 5 minutes), and 10 minutes after sublingual administration of glycerol trinitrate (GTN; 0.4 mg). Because of small but significant effects, the initial 7 volunteers were recalled for a separate protocol with placebo treatment, together with a second group in whom a true placebo control was performed. The local ethics committee approved this study, and informed consent was obtained.

Platelet Preparation and Flow Cytometry
Whole blood was diluted with PBS (free of Ca²⁺ and Mg²⁺ and enriched with d-glucose 5.5 mmol/L and 0.5% bovine albumin) immediately after collection and incubated with an FITC-labeled anti-fibrinogen antibody (WAK-Chemie) for determination of platelet binding of fibrinogen, anti-human FITC-conjugated anti–P-
selectin (CD62P) antibody for P-selectin, anti-human FITC-conjugated anti-CD154 antibody for CD40 ligand, or anti-human FITC-conjugated anti-CD63 antibody for surface expression of glycoprotein 53 (Becton Dickinson) for 10 minutes at room temperature. Control samples were incubated with FITC-conjugated isotype IgG in parallel to control for nonspecific binding. Platelets were fixed with methanol-free formaldehyde (1.5%) for 10 minutes and analyzed in a Becton Dickinson FACSCalibur at a low flow rate. The platelet population was identified on its forward and side scatter distribution, and 20,000 events were analyzed for mean fluorescence with CELLQuest software, version 3.1f, as initially described. Platelet specificity was documented by CD41 and CD42 positivity.

VASP phosphorylation was evaluated with an FITC-labeled antibody (5C6 antibody 50 μg/mL; Nanotools) after fixation of the blood samples by methanol-free formaldehyde (1.5%, 5 minutes). Samples were diluted with PBS and allowed to permeabilize for 10 minutes after the addition of Triton X100 (0.2% final). Nonspecific binding was controlled for through the use of a blocking peptide as described before and arbitrarily adjusted to a mean fluorescence of 10. The investigator performing flow cytometry was blinded to treatment.

Flow-Mediated Dilation
Assessment of flow-mediated dilation was performed by MRI on a 1.5-T clinical MR scanner (Siemens Sonata). MR measurement of the brachial artery area was performed at baseline and after reactive hyperemia was induced by release of a forearm cuff inflated to suprasystolic pressure for 4.5 minutes. For assessment of endothelial-independent brachial artery dilatation, MR data acquisition was repeated before and 3 minutes after GTN. Cardiac gated TrueFISP cine images of the brachial artery were acquired with the following parameters: repetition time/echo time, 56/3 ms; flip angle, 66°; field of view, 117×77 mm; matrix, 384×252; segments, 16; and phases, 11 to 19, depending on heart rate.

Statistical Analysis
Values are mean±SEM. Statistical evaluation of platelet and hemodynamic parameters was performed by repeated-measures 2-way ANOVA, followed by a Tukey post-hoc test. P<0.05 was considered statistically significant.

Results
NOS inhibition by L-NMMA was confirmed by the expected modest increase in blood pressure after intravenous administration (basal, 117±3/69±2 mm Hg; L-NMMA, 129±2 [P<0.05]/82±2 [P<0.01 versus basal] mm Hg; n=12) and decrease in flow-mediated brachial artery dilation (basal, 14.3±2.4%; L-NMMA, 6.4±1.0%; n=6; P<0.05). Restoration of NOS bioactivity by sublingual GTN returned blood pressure to baseline levels (111±3 [P<0.05]/65±2 [P<0.01 versus L-NMMA] mm Hg) and caused a brachial artery dilation response (44.1±2.9%). NO bioactivity in platelets determined by VASP phosphorylation was significantly reduced in all individuals after 30 minutes of NOS inhibition by L-NMMA injection. GTN reversed the reduction in VASP phosphorylation (Figure 1).

NOS inhibition resulted in enhanced platelet binding of fibrinogen (Figure 2A through 2C). NOS inhibition significantly increased platelet degranulation demonstrated by enhanced surface expression of P-selectin (CD62P; Figure 2D through 2F), CD40 ligand (CD154; Figure 2G through 2I), and glycoprotein 53(CD63; Figure 2J through 2L) in all individuals. Administration of GTN restored all parameters of platelet activation to baseline levels. Placebo controls were performed in the same subjects, and no changes in blood pressure or platelet activation were observed.

Discussion
In this study, we demonstrate that acute reduction in NO bioavailability in vivo rapidly increases platelet activation, which is immediately reversed by the NO donor GTN, demonstrating a direct and rapid relationship between NO bioavailability and human platelet function in vivo. Our observations suggest that platelet activation in healthy individuals in vivo is suppressed by tonic NO production, resulting in immediate platelet activation when NO production is inhibited.

Acute inhibition of systemic NO synthesis enhanced fibrinogen binding, which was rapidly reversed by GTN, demonstrating that the fast action of NO causing inhibition of glycoprotein IIB/IIIa in vivo contributes to regulation of platelet activation as described before under in vitro conditions. We found increased surface expression of P-selectin...
Figure 2. Platelet-bound fibrinogen (A through C) and surface expression of P-selectin (CD62P; D through F), CD40 ligand (CD154; G through I), and glycoprotein 53 (CD63; J through L) on platelets from healthy volunteers at baseline, after intravenous NOS inhibition with L-NMMA, and after sequential application of GTN. Data from 12 different volunteers are shown as mean fluorescence for every individual sample (A, D, G, J), representative flow cytometry histogram (B, E, H, K), and ratio of antigen-positive platelets to total platelets (±SEM; C, F, I, L). *P<0.05,**P<0.01 vs baseline and GTN.
and CD40 ligand in platelets after short-term systemic NOS inhibition. P-selectin can participate in platelet adhesion to the endothelium and is responsible for platelet leukocyte adhesion, which can adversely influence the progression of cardiovascular diseases with reduced NO bioavailability. The expression of CD40 ligand on the platelet surface enables adhesion to the endothelium. In several cardiovascular diseases such as diabetes, heart failure, and coronary artery disease, systemic NO formation/bioavailability is impaired and platelet activation is enhanced. As reported previously, chronic NOS inhibition increased platelet activation when used as a chronic model of hypertension. In most of these disease states, platelet activation has been attributed to factors other than reduced NO bioavailability, including endothelial activation with enhanced expression of adhesion molecules, cytokine release, leukocyte activation, or neurohumoral factors. The rationale for our use of healthy volunteers was to explicitly exclude potential inflammatory platelet-activating mediators and to assess whether the acute loss of NO under physiological conditions was sufficient to cause platelet activation. Because NO acts as an inhibitor of platelet activation, the results of our study indicate that some platelet-activating mediators are present under physiological conditions, resulting in a balance between platelet-activating and platelet-inhibiting substances (including NO).

To evaluate the bioactivity of NO toward platelets, we assessed platelet VASP phosphorylation in parallel with markers of platelet activation. VASP phosphorylation reflects the integrity of platelet-inhibitory pathways, including the predominant NO/cGMP pathway. When systemic NO formation was inhibited in our study, platelet VASP phosphorylation was reduced, indicating fewer NO-mediated effects on platelets. VASP phosphorylation affects initial sequences in platelet adhesion and activation, thus, decreased VASP phosphorylation after NOS inhibition could directly contribute to the observed changes in platelet activation. The observed activation was similar to that achieved by in vitro stimulation of human platelets with 30 μmol/L ADP. When VASP phosphorylation was normalized after GTN, surface P-selectin and CD40 ligand returned to baseline conditions.

The results of our study differ from an earlier study with infusion of L-NMMA. In that study, a cumulative infusion of L-NMMA (6 to 9 mg/kg) did not result in enhanced platelet activation or susceptibility to platelet agonists in vitro. In our study, L-NMMA was given as a fractionated bolus, which might have resulted in earlier and more extended complete blockade of NOS, explaining the reduced NO action on platelets as demonstrated by reduced VASP phosphorylation and thus increased platelet activation. The rapid regulation of platelet activation by NO in vivo may not be detectable after the time taken to prepare platelets for in vitro assays because many in vitro experiment is performed in conditions lacking endothelial NO.

Conclusions

The present study demonstrates that acute changes in systemic NO bioavailability rapidly modulate platelet activation under in vivo conditions. Reduced NO bioavailability in many cardiovascular diseases might directly contribute to platelet activation in these patients and thereby influence disease progression.

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