Impaired Platelet Production of Nitric Oxide Predicts Presence of Acute Coronary Syndromes

Jane E. Freedman, MD; Brian Ting, BS; Beth Hankin, BA; Joseph Loscalzo, MD, PhD; John F. Keaney, Jr, MD; Joseph A. Vita, MD

Background—Thrombus formation within a coronary vessel is the acute precipitating event in most acute coronary syndromes. Recently, constitutive nitric oxide synthase (cNOS) has been identified in human platelets, and platelet-derived nitric oxide has been shown to inhibit platelet recruitment after aggregation. However, its role in regulating platelet responses under normal or pathologic conditions has not yet been elucidated.

Methods and Results—We examined nitric oxide (NO) production by platelets isolated from 87 patients undergoing coronary angiography, 37 with stable angina and 50 with unstable angina or a myocardial infarction within 2 weeks. After stimulation with 5 μmol/L ADP, platelet aggregation and NO production were simultaneously measured with an NO-selective microelectrode adapted for use in a standard platelet aggregometer. Mean (±SEM) platelet-derived NO production was 1.78 ± 0.36 pmol/10⁸ and 0.26 ± 0.05 pmol/10⁸ platelets in coronary patients with stable angina and acute coronary syndromes, respectively (P = 0.001). By logistic regression analysis, heparin treatment (odds ratio 6.6, CI 1.9 to 22.8, P = 0.003), lower platelet-NO production (odds ratio 4.0, CI 1.3 to 11.5, P = 0.01), and extent of atherosclerosis (odds ratio 1.5, CI 1.1 to 2.0, P = 0.02) were independent predictors of an acute coronary syndrome. In the subset of patients with angiographic evidence of atherosclerosis (n = 83), logistic regression demonstrated that platelet NO production (odds ratio 3.9, CI 1.3 to 11.1, P = 0.01) and heparin treatment (odds ratio 6.4, CI 1.9 to 22.0, P = 0.004) were independent predictors of an acute coronary syndrome, whereas extent of atherosclerosis was not.

Conclusions—In summary, aggregating platelets from patients with acute coronary syndromes produce less NO. Since platelet aggregation and thrombus formation are implicated in unstable angina and myocardial infarction, impaired platelet-derived NO production may contribute to the development of acute coronary syndromes. (Circulation. 1998;98:1481-1486.)

Key Words: platelets • nitric oxide • thrombosis • coronary disease

Thrombus formation within a coronary vessel is the precipitating event in myocardial infarction and unstable angina, as documented by angiographic and pathologic studies. Rupture of atheromatous plaque in relatively mildly stenosed vessels and subsequent occlusive thrombus formation is believed to be responsible for most acute coronary syndromes. Both superficial and deep intimal injury lead to the adherence of platelets to the subendothelium and, subsequently, platelet activation. Once activated, platelets further stimulate thrombus formation and recruit additional platelets by releasing ADP and serotonin, producing thromboxane A₂, and promoting surface thrombin generation. Increased platelet-derived thromboxane and prostaglandin metabolites have been found in acute coronary syndromes, providing biochemical support for platelet activation. The importance of platelet activation in acute coronary syndromes is further supported by the clear clinical benefit of treatment with aspirin.

Platelet activation and recruitment are tightly regulated by products of the endothelium, including prostacyclin and NO (nitric oxide). NO inhibits platelet adhesion and aggregation, and prevents thrombosis in a model of endotoxin-induced glomerular damage. Endothelial production of bioactive NO is impaired in atherosclerosis and in the presence of coronary risk factors including hypercholesterolemia, diabetes mellitus, cigarette smoking, and hypertension. Furthermore, there is evidence that loss of endothelium-derived NO contributes to the pathogenesis of acute coronary syndromes.

Both constitutive and inducible nitric oxide synthase (NOS) have been identified in human platelets and megakaryocytoid cells, and studies report NO release from aggregating platelets. Platelet aggregation is enhanced by incubation with inhibitors of NOS and inhibited by incubation with the NOS substrate L-arginine. Importantly, whereas platelet-derived NO appears to inhibit the primary aggregation response modestly, NO release from activated human platelets markedly inhibits platelet recruitment and thus may limit progression of intra-arterial thrombosis.
vivo, systemic infusion of the NOS inhibitor L-N\textsuperscript{-}monomethyl arginine citrate (L-NMMA) causes a reduction in bleeding time without change in vessel tone\textsuperscript{28} and enhances platelet reactivity to various agonists,\textsuperscript{29} supporting the clinical relevance of platelet-derived NO. Although the role of endothelium-derived NO has been extensively characterized, the relation between platelet-derived NO production and cardiovascular disease has not been previously investigated. Since coronary thrombosis is the precipitating event in most acute coronary syndromes and loss of platelet-derived NO promotes platelet recruitment, we sought to examine the relation between platelet NO production and clinical presentation in patients with coronary artery disease.

### Methods

**Patients**

Consecutive patients referred to Boston Medical Center for cardiac catheterization were enrolled in accordance with the policies of the Institutional Review Board. Medications, including aspirin, were not discontinued. At the beginning of the catheterization, a 45-mL blood sample was collected for plasma and platelet analysis.

A research nurse identified the presence of the following risk factors: (1) age, (2) male sex, (3) clinical history of diabetes (fasting blood glucose >140 mg/dL or treatment with insulin or an oral hypoglycemic agent), (4) clinical history of hypertension (blood pressure >90 mm Hg diastolic or treatment for hypertension), (5) cigarette smoking (pack-years and most recent cigarette), (6) clinical history of hypercholesterolemia, and (7) family history of coronary disease (first-degree relative with myocardial infarction or cardiac death before age 55). Patients were also questioned about peripheral vascular disease, ethanol use, medications, and multivitamin use. Total cholesterol, HDL cholesterol, and triglycerides were measured in the Boston Medical Center clinical laboratory. LDL cholesterol was calculated with the Friedewald formula.\textsuperscript{30}

**Assessment of Extent of Coronary Atherosclerosis**

Coronary angiograms were analyzed off-line in a blinded fashion with the use of digital calipers to measure stenosis severity, and stenosis was defined as a dichotomous variable: if a stenotic lesion was >50%, that vessel was counted as stenosed. Patients were ranked as having 0- to 3-vessel disease. Extent of atherosclerosis was also quantified using the Hamsten extent score,\textsuperscript{31} which reflects the extent of early disease and is expressed on a scale of 0 (no disease) to 9 (extensive atherosclerosis in each of 15 coronary segments).

**Classification of Clinical Disease Activity**

We obtained a detailed angina history and reviewed the medical record for evidence of unstable angina as defined by Braunwald\textsuperscript{32} or myocardial infarction as indicated by the presence of typical symptoms, ischemic ECG changes, and a diagnostic elevation of CK\textsubscript{MB} fraction. We categorized each subject as stable or having an acute coronary syndrome, depending on the presence or absence of unstable angina or a myocardial infarction within 2 weeks of study. Subjects were categorized by investigators blinded to platelet NO results.

**Preparation of Samples**

The blood was centrifuged (150g, 15 minutes, 22°C) and the supernatant, representing 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.\textsuperscript{33} Platelet counts were determined with the use of a Coulter Counter, model ZM (Coulter Electronics).

**Measurement of Platelet NO Production and Aggregation**

We adopted a NO-selective\textsuperscript{34} micro-electrode (Inter Medical Co, Ltd) for use in a standard platelet aggregometer (Payton Associates) to monitor platelet NO production and aggregation simultaneously, as previously described.\textsuperscript{35} Platelet NO production was quantitated as the integrated signal detected by the micro-electrode after platelet activation with 5 μmol/L ADP. Aggregation of GFP was monitored with a standard naphelometric technique as previously described.\textsuperscript{36}

Platelet NO production was initially studied in 41 normal controls (age 30±2 years) and ranged from 0.5 to 15.2 pmol/10\textsuperscript{8} platelets (mean 3.3±1.4 pmol/10\textsuperscript{8} platelets). To assess interassay reproducibility, GFP was prepared from 10 normal control subjects (age 31±2 years) on 2 different days. Platelet NO production in this population ranged from 0.5 to 14.7 pmol/10\textsuperscript{8} platelets (mean 3.6±1.0 pmol/10\textsuperscript{8} platelets). For each donor, the average difference in between-day determinations of NO production was 0.36±0.36 pmol/10\textsuperscript{8} platelets and the average within-day difference between determinations of NO production was 0.50±0.25 pmol/10\textsuperscript{8} platelets for each donor (P=NS). In each subject, the NOS inhibitor N\textsuperscript{-}NAME nitroarginine methyl ester (L-NNAME) inhibited at least 80% of the NO signal, confirming its dependence on NO production. The limit of detection for the assay is 0.05 pmol/10\textsuperscript{8} platelets.

**Statistical Analysis**

Clinical characteristics, coronary risk factors, angiographic findings, medication use, platelet function, and platelet NO production for the stable and acute coronary syndrome groups were compared with the use of the χ\textsuperscript{2} test for proportions, the 2-sample t tests for normally distributed continuous variables, and the Mann-Whitney U test for continuous variables with a nonnormal distribution. Normality was determined with the Kolmogorov-Smirnov algorithm. Platelet NO production had a nonnormal distribution. To determine which patient characteristics were associated with low platelet NO production, patients were categorized as having platelet NO production less than, greater than, or equal to the median value, and the 2 groups were compared with the use of the χ\textsuperscript{2} test or the 2-sample t test as appropriate. Logistic regression was used to identify independent predictors of an acute coronary syndrome. Statistical significance was accepted at the P<0.05 level. Analyses were completed using SPSS for Windows, Release 6.0 (SPSS, Inc). All data are expressed as mean±SEM.

**Results**

**Patient Characteristics**

A total of 87 subjects were enrolled in the study. Fifty subjects had an acute coronary syndrome (unstable angina or myocardial infarction) and 37 subjects did not. The clinical characteristics, medications, and angiographic findings for the 2 groups are displayed in Table 1. As shown, the subjects with an acute coronary syndrome had more extensive coronary atherosclerosis and were more likely to be receiving nitrate or heparin treatment than were the stable subjects.

**Platelet NO Production**

For all patients, platelet NO production after activation by ADP ranged from 0.00 to 9.02, with a mean of 0.90±0.18 pmol/10\textsuperscript{8} platelets. The values for platelet NO production were not normally distributed and had a median value of 0.30 pmol/10\textsuperscript{8} platelets. As shown in representative tracings in Figure 1 and summarized in Figure 2, production of NO by platelets was lower in patients with an acute coronary syndrome than in stable patients. Extent of platelet aggregation and platelet count after gel filtration were similar in the 2 groups (Table 2). In addition, patients with significant
coronary artery disease (stenosis ≥50%, n=73) and patients with no significant coronary artery disease (n=14) had platelet NO levels of 0.62±1.5 pmol/10^8 platelets and 2.4±1.7 pmol/10^8 platelets, respectively (P<0.0001). Platelet NO levels were not significantly different between patients with unstable angina and patients with myocardial infarction.

To investigate whether low platelet NO production is an independent predictor of an acute coronary syndrome, we first identified potential confounders by comparing the clinical characteristics listed in Table 1 in the groups of patients with platelet NO production greater than and less than, or equal to the median value. Patients with low platelet NO production were more likely to be receiving heparin (49% versus 27%, P=0.04) and were more likely to be receiving nitrate therapy (72% versus 50%, P=0.03). There was a trend for a higher atherosclerosis extent score in the patients with low platelet NO production (3.6±0.3) compared with patients with high platelet NO production (2.8±0.2, P=0.06). No other clinical marker differed significantly according to level of NO production.

To identify the independent predictors of an acute coronary syndrome, a logistic regression model was constructed using these 4 variables: category of platelet NO production, heparin treatment, nitrate treatment, and atherosclerosis extent score. As shown in Table 3, the independent predictors of an unstable coronary syndrome were low platelet NO production, atherosclerosis extent score, and the presence of heparin treatment.

No subject with angiographically normal arteries had an acute coronary syndrome, and we considered the possibility that the association between extent of atherosclerosis and an acute coronary syndrome was driven by this finding. Therefore, we investigated predictors of an acute coronary syndrome in the 83 patients with atherosclerosis extent score >0. In this group of patients with proven coronary artery

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![Figure 1. Effect of platelet activation on platelet NO production in patients with stable (left) and unstable (right) ischemic coronary syndromes. Shown are representative tracings of NO production by gel-filtered platelets stimulated with 5 µmol/L ADP as described in “Methods.”](image)

![Figure 2. Platelet NO production in patients with stable and acute coronary syndromes. Platelet NO production after activation with ADP was significantly decreased (P=0.0001) in patients with acute coronary syndromes as compared with patients with stable disease.](image)
Heparin treatment (odds ratio 6.6, CI 1.9 to 22.0, \( P = 0.004 \)) were independent predictors of an acute coronary syndrome, whereas extent of atherosclerosis was not.

### Discussion

In this study, we evaluated stimulated platelet NO production in a group of 87 patients referred for cardiac catheterization. The patients with an acute coronary syndrome within 2 weeks of study had markedly lower platelet NO production than patients with stable or no angina. Acute coronary syndrome patients, not surprisingly, also were more likely to be receiving heparin and nitrate therapy and had more extensive coronary atherosclerosis. By logistic regression analysis, low platelet NO production, heparin therapy, and extent of coronary atherosclerosis were independent predictors of an acute coronary syndrome whereas nitrate treatment was not. Platelet NO production but not extent of atherosclerosis was an independent predictor of an acute coronary syndrome in the subset of patients with coronary artery disease, suggesting that once atherosclerosis is present, factors other than the extent of disease are important. Thus, low platelet NO production could be a contributing mechanism in the pathology of acute coronary syndromes.

In the present study, heparin therapy was associated with both lower platelet NO production and acute coronary syndromes, raising the possibility that a confounding effect of heparin treatment might explain the strong relation between low platelet NO production and acute coronary syndromes. In high concentrations, heparin is known to decrease NO production in endothelial cells,\(^37\) but there currently is no evidence for a direct effect of heparin on NO production in platelets. A direct heparin effect is unlikely to explain the present findings because platelet NO production remained an independent predictor of acute coronary syndromes after controlling for heparin treatment. Nitrate treatment also has the potential to affect platelet NO production. For example, it is known that NO can downregulate cNOS activity in disease, logistic regression demonstrated that platelet NO production (odds ratio 3.9, CI 1.3 to 11.1, \( P = 0.01 \)) and heparin treatment (odds ratio 6.4, CI 1.9 to 22.0, \( P = 0.004 \)) were independent predictors of an acute coronary syndrome, whereas extent of atherosclerosis was not.

### Table 3. Multivariate Analysis for Predictors of Acute Coronary Syndromes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin treatment</td>
<td>6.6</td>
<td>1.9–22.8</td>
<td>0.003</td>
</tr>
<tr>
<td>Low platelet NO production</td>
<td>4.0</td>
<td>1.3–11.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Atherosclerosis extent score</td>
<td>1.5</td>
<td>1.1–2.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Nitrate treatment</td>
<td>1.5</td>
<td>0.5–4.6</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Impairment of endothelium-derived nitric oxide has been extensively characterized in patients with atherosclerosis,\(^{47}\) risk factors for coronary artery disease,\(^{17}\) and acute coronary syndromes;\(^{18,19}\) however, platelet NO production has not been previously studied in patients with coronary disease. By direct measurement of NO production, the present study indicates that impairment of platelet-derived NO may also be linked to the pathogenesis of acute coronary syndromes. Although platelet NO release does not have a major effect on

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**TABLE 2. Platelet Parameters in Patients Referred for Cardiac Catheterization**

<table>
<thead>
<tr>
<th></th>
<th>Stable (n=37)</th>
<th>Acute Coronary Syndrome (n=50)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count of GFP (1000/μL)</td>
<td>170±12</td>
<td>149±11</td>
<td>0.21</td>
</tr>
<tr>
<td>Extent of aggregation (%)</td>
<td>59±4</td>
<td>55±3</td>
<td>0.34</td>
</tr>
</tbody>
</table>

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Evidence for a direct effect of heparin on NO production in platelets. A direct heparin effect is unlikely to explain the strong relation between platelet NO production and extent of ex vivo platelet aggregation.

Only one prior study has examined platelet NO production in human subjects, and it demonstrated lower values in chronic cigarette smokers compared with healthy, nonsmoking control subjects.\(^{41}\) Although we found that platelet NO production was similar in smokers and nonsmokers, differences in the study populations likely explain these seemingly disparate findings. Unlike the subjects studied by Ichiki and colleagues,\(^{41}\) the present study examined older patients, and the majority of these patients had symptomatic coronary artery disease. Both smokers and nonsmokers had relatively low platelet NO production compared with historical normal controls from our laboratory. As in the present study, Ichiki and colleagues demonstrated no relation between platelet NO production and extent of ex vivo platelet aggregation.

The mechanism for decreased NO release from platelets in patients with acute coronary symptoms has not yet been established. A prominent feature of both abnormal platelet function and dysfunctional endothelium-dependent vasodilation in the setting of cardiovascular disease is increased oxidative stress. There is ample clinical evidence to suggest that oxidative stress and antioxidant status is important in normal platelet function. For example, alteration of the platelet redox status causes increased production of reactive oxygen species, including superoxide.\(^{42}\) Metabolism of reactive oxygen species by antioxidants may also alter platelet function. In patients with coronary artery disease, decreased plasma and platelet antioxidant activity is associated with increased platelet aggregability.\(^{43}\) In a recent clinical study, vitamin E supplementation was associated with increased hemorrhagic stroke.\(^{44}\) In addition, we have shown that the antioxidant enzyme glutathione peroxidase potentiates the inhibition of platelet function by NO through metabolism of reactive oxygen species\(^{45}\) and that impairment of this process can lead to a clinical thrombotic disorder.\(^{46}\)

Impairment of endothelium-derived nitric oxide has been extensively characterized in patients with atherosclerosis,\(^{47}\) risk factors for coronary artery disease,\(^{17}\) and acute coronary syndromes;\(^{18,19}\) however, platelet NO production has not been previously studied in patients with coronary disease. By direct measurement of NO production, the present study indicates that impairment of platelet-derived NO may also be linked to the pathogenesis of acute coronary syndromes. 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 recruitment of platelets to the growing thrombus.26 Since platelet recruitment is a primary means of thrombus propagation, it is reasonable to speculate that loss of platelet NO could increase the risk for development of unstable angina or acute myocardial infarction where coronary thrombosis is a primary event. The findings of the present study support the clinical relevance of this mechanism.

In summary, low platelet NO production was independently associated with the presence of an acute coronary syndrome, a finding that could not be attributed to concurrent medical therapy or other clinical and coronary risk factors. It should be acknowledged that the present study is cross-sectional in nature, and we cannot exclude the possibility that some unmeasured confounding factor associated with acute myocardial infarction or unstable angina accounts for our findings. However, our results may support the hypothesis that impaired platelet-derived NO production contributes to the development of acute coronary syndromes in patients with coronary artery disease and may be a suitable target for therapy.

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References

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