Platelet-Derived Nitric Oxide and Coronary Risk Factors

Hisao Ikeda, Yoshinori Takajo, Toyoaki Murohara, Kazuya Ichiki, Hisashi Adachi, Nobuya Haramaki, Atsushi Katoh, Tsutomu Imaizumi

Abstract—Platelet aggregation is inhibited through a negative feedback mechanism by the L-arginine/nitric oxide (NO) pathway found in platelets themselves. We have shown that long-term smoking impairs the bioactivity of platelet-derived NO (PDNO), resulting in an increased platelet aggregability. However, little is known about the relation between other coronary risk factors and PDNO release. Accordingly, this study was undertaken to examine whether other coronary risk factors are related to the impairment of PDNO bioactivity. We measured collagen-induced PDNO release with an NO-selective electrode in 61 subjects (mean age 47 years, range 24 to 74 years) who underwent complete physical and laboratory examinations. There was a significant inverse correlation between PDNO release and the number of coronary risk factors ($r = -0.61, P < 0.001$). Univariate analysis showed a significant inverse correlation between PDNO release and age ($r = -0.33, P < 0.01$), mean arterial pressure ($r = -0.40, P < 0.002$), total cholesterol level ($r = -0.31, P < 0.02$), and LDL-cholesterol level ($r = -0.33, P < 0.02$). PDNO release was significantly lower in long-term smokers than in nonsmokers ($P < 0.001$). With multiple stepwise regression analysis, PDNO release correlated significantly and independently ($r^2 = 0.51$), with smoking ($F = 37.8$), age ($F = 7.1$), and mean arterial pressure ($F = 5.1$). Thus, we demonstrated that coronary risk factors are associated with an impairment of PDNO release by human platelets. Our findings may contribute to the understanding of the pathophysiological link between coronary risk factors and atherothrombotic disease. (Hypertension. 2000;35:904-907.)

Key Words: platelets | nitric oxide | risk factors | atherothrombosis

Platelets possess the functional L-arginine/nitric oxide (NO) pathway via constitutive NO synthase. Platelet-derived NO (PDNO) increases the intraplatelet level of cGMP and inhibits platelet aggregation. This modulation of platelet aggregation via the L-arginine/NO pathway is recognized as a negative-feedback mechanism to inhibit aggregation. Using an NO-selective electrode, we and others directly measured PDNO release during platelet aggregation, which was potentiated by L-arginine and attenuated by inhibitors of NO synthase, indicating the existence of an L-arginine/NO pathway in human platelets. PDNO has been shown to play a functional role in the inhibition of not only platelet activation but also platelet recruitment after aggregation. Recently, we have shown that long-term smoking impairs PDNO release, resulting in an increased platelet aggregability. However, little is known about the relation between other coronary risk factors and PDNO release. Accordingly, the present study was undertaken to investigate the hypothesis that other coronary risk factors impair platelet function and thereby affect PDNO release in subjects with major risk factors.

Methods

Study Subjects
The study population consisted of 61 subjects who agreed to participate in the study. The subjects underwent complete routine physical and laboratory examinations, and their complete anamnesis was obtained. Of the 61 subjects, 42 were healthy volunteers, 12 had stable effort angina, and 7 had previous myocardial infarction. Medications, including long-acting nitrates, calcium channel blockers, $\beta$-blockers, and ACE inhibitors, were withheld for $\geq 24$ hours, and aspirin was withheld for $\geq 1$ week before the study. None of the patients had received cholesterol-lowering agents. Patients with acute coronary syndromes, valvular heart diseases, or heart failure were excluded from the study. The present protocol was approved by our institutional ethic committee, and informed consent for the study was obtained from all patients.

Definition of Coronary Risk Factors
Coronary risk factors in this study were determined according to the Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure report. Hypertension was defined as a systolic blood pressure of $\geq 140$ mm Hg, a diastolic blood pressure of $\geq 90$ mm Hg, or both. Hypercholesterolemia was defined as a total serum cholesterol level $\geq 220$ mg/dL, which was measured with an enzymatic method. None of the patients had documented familial hyperlipidemia. Subjects...
who smoked $\geq$15 cigarettes per day for $\geq$5 years were considered to be long-term smokers. Long-term smokers were asked to abstain from smoking for $\geq$120 minutes before the study to avoid the acute effects of smoking on platelet function. Family history was considered positive if a first-degree relative had clinical evidence of coronary artery disease (angina pectoris or myocardial infarction) at the age of $\leq$60 years. None of the patients had a history of insulin-dependent diabetes mellitus or elevated blood glucose. A composite risk factor score was calculated for each subject, with 1 point given for each of the following: age of $\geq$60 years, current smoker, hypertension, hypercholesterolemia, and family history of coronary artery disease.

**Preparation of Washed Platelets**
Platelet suspensions were prepared according to a previously described method. Briefly, 20 mL of blood from all subjects was collected through venipuncture into a plastic tube containing 3.15% trisodium citrate (1:9 v/v) and prostacyclin (2 $\mu$g/mL; Sigma Chemical Co) and then centrifuged at 250g for 20 minutes at 22°C. The obtained supernatant, platelet-rich plasma, was centrifuged again at 900g for 10 minutes at 22°C after the addition of prostacyclin (300 ng/mL). The platelet pellet was resuspended in 5 mL of Ca$^{2+}$- and Mg$^{2+}$-free Tyrode’s solution (pH 7.4) containing prostacyclin (300 ng/mL) and centrifuged at 800g for 10 minutes at 37°C. The platelet pellet was resuspended in Tyrode’s solution containing Ca$^{2+}$ and Mg$^{2+}$. The platelet counts were then adjusted to the range of 1 to 2 x 10$^9$ platelets/µL in Tyrode’s solution. Tyrode’s solution consisted of the following components (in mmol/L): NaCl 136, KCl 2.7, NaHCO$_3$ 12, NaH$_2$PO$_4$ 1, CaCl$_2$ 1.8, MgCl$_2$·6H$_2$O 1, glucose 5.5, and HEPES 5, pH 7.4.

**Measurements of PDNO With an NO-Specific Electrode**
We measured PDNO with an NO meter (model NO-501; Inter Medical Co) that measures the picomamper-order redox current between the working electrode and the counter electrode, as previously described. Briefly, the working electrode consisted of a platinum/iridium alloy wire (OD 0.2 mm) coated with a 3-layer membrane to avoid the KCl membrane, NO-selective resin, and normal silicone membrane. The counter electrode was made of carbon fiber (OD 0.5 mm). The NO meter and electrodes were placed into an electromagnetic shield box to avoid the electrical perturbation of the electrodes. The two electrodes were placed in a chamber containing washed platelet suspensions. After the addition of collagen (3 $\mu$g/mL), the working electrode was supplied with +0.6 V for the electrochemical oxidation of NO diffusing through the membrane to avoid currents from possible contaminants of oxygen because electrode responses to oxygen changes were observed at voltages of $\leq$+0.4 V. The obtained electrical current was considered to be an index of NO release.

**Statistical Analysis**
Data are expressed as mean±SD. The number of risk factors was considered a continuous variable and compared with PDNO release with linear regression analysis. Repeated measures ANOVAs was applied for multiple comparisons. Univariate analysis of the effects of each risk on PDNO release was performed with linear regression for continuous variables (age; systolic, diastolic, and mean blood pressures; and total, HDL-, and LDL-cholesterol levels) and 1-way ANOVA for categorical variables (gender, smoking, family history of coronary artery disease, evidence of coronary artery disease). Then, the interaction between various risk characteristics and PDNO release was examined with multiple stepwise regression analysis. Statistical significance was considered at the level of $P<0.05$.

**Results**
**Clinical Characteristics of the Subjects**
The subjects consisted of 52 men and 9 women (30 nonsmokers and 31 smokers, mean age 47 years, range 24 to 74 years).

![Figure 1. Plot of the relation between PDNO release and the number of coronary risk factors. PDNO release exhibited a significant ($r=-0.61, P<0.001$) inverse correlation with the number of risk factors. $P<0.05$ vs risk 0.](image)

The averaged systolic, diastolic, and mean arterial pressures were 127±15, 76±10, and 93±10 mm Hg, respectively; 15 subjects had hypertension. Five subjects had a positive family history of coronary artery disease. The average levels of total, HDL-, and LDL-cholesterol and triglycerides were 192±27, 118±27, 48±13, and 128±56 mg/dL, respectively; 12 subjects had hypercholesterolemia.

**PDNO and Coronary Risk Factors**
Mean PDNO was 9.6±4 pA (range 3.5 to 22 pA). The mean number of risk factors was 1.4±1.1, and the composite risk factor score was 0 in 17, 1 in 17, 2 in 17, 3 in 8, and 4 in 2 subjects. PDNO release was significantly lower in subjects with risk factors (8.3±2.4, 8.9±2.7, 6.4±3.1, and 4.5±0.7 pA for subjects with 1, 2, 3, and 4 risk factors, respectively) than in subjects without risk factors (13.6±3.6 pA) ($P<0.05$). PDNO release exhibited a significant inverse correlation with the number of risk factors ($r=-0.61, P<0.001$; Figure 1). Univariate analysis showed a significant inverse correlation between PDNO release and age ($r=-0.33, P<0.01$; Figure 2A), systolic blood pressure ($r=-0.38, P<0.005$), diastolic blood pressure ($r=-0.34, P<0.01$), mean arterial pressure ($r=-0.40, P<0.01$; Figure 2B), total cholesterol level $r=0.33$, $P<0.01$.
Multiple Stepwise Regression Analysis for Determinant of PDNO

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette smoking</td>
<td>−4.492</td>
<td>37.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>−0.071</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
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</tr>
<tr>
<td>Constant</td>
<td>23.33</td>
<td></td>
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</tbody>
</table>

Gender 0.01 NS
Positive family history 0.09 NS
Coronary artery disease 1.39 NS
Total cholesterol 1.89 NS
Triglycerides 0.89 NS
HDL-cholesterol 0.04 NS
LDL-cholesterol 1.25 NS

$r^2=0.51$.

Discussion

In the present study, the most important findings were that (1) the significant inverse correlation was found between PDNO release and the number of risk factors and that (2) PDNO release independently correlated with smoking, aging, and hypertension. Thus, our findings suggest that impairment of PDNO release by platelets is associated with coronary risk factors in humans.

It has been shown that an NO-selective electrode is specifically capable of measuring real-time release of NO from endothelial cells\(^{11,13,14}\) and platelets,\(^{6,15}\) We also previously showed that the electrode used in this study was sufficiently sensitive to detect the release of NO from aggregated platelets on the basis of the following findings.\(^5\)

First, S-nitroso-N-acetyl-dl-penicillamine, a direct NO donor, dose-dependently increased the electrical current. Second, the electrical current showed a high correlation with collagen concentrations from 1 to 5 µg/mL (r=0.94). Third, the collagen-induced electrical current and intraplatelet levels of cGMP were increased by L-arginine and attenuated by \(\text{N}^\text{G}\)-monomethyl-L-arginine, an inhibitor of NO synthesis. Fourth, a good correlation was found between collagen-induced intraplatelet cGMP and the electrical current (r=0.73). Thus, we confirmed that the changes in the electrical current reflect the amount of NO released through the L-arginine/NO pathway in aggregated platelets.

We previously reported that long-term smoking impairs PDNO release.\(^5\) The same findings were obtained in this study. We further examined whether other major risk factors are associated with impaired PDNO release during platelet aggregation. A significant inverse correlation was observed between PDNO release and the number of risk factors. Univariate analysis showed that PDNO release was inversely related to risk factors such as aging, hypertension, and hypercholesterolemia. Thus, our findings suggest not only that individual risk factors impair PDNO release but also that PDNO release is further impaired when the number of risk factors increases. When the multiple stepwise regression model was used to predict PDNO release with age, gender, cholesterol level, blood pressure, smoking, and family history as independent variables, PDNO release was found to be independently related to smoking, aging, and hypertension. Most importantly, PDNO release showed the strongest negative correlation with smoking. Accordingly, among major risk factors, smoking is the most powerful factors affecting PDNO release. Taken together, our findings may suggest that the L-arginine/NO pathway, as a negative feedback mechanism to inhibit platelet aggregation, is impaired in subjects with major risk factors, leading to an increased platelet aggregability and the development of atherothrombotic disease.

In the present study, the presence of coronary risk factors was associated with an impairment of PDNO release in human platelets. These risk factors have been shown to impair endothelium-dependent vasodilation\(^{16-18}\) and to reduce bioactivity of NO\(^9\) in the human coronary arteries. On the bases of the present and other studies, coronary risk factors may impair the L-arginine/NO pathway in both endothelial cells and platelets. Epidemiological studies have demonstrated that exposure to multiple risk factors increases the occurrence of atherosclerosis and thrombosis.\(^{20,22}\) Because endogenous NO has antiatherothrombotic actions,\(^9,23\) our findings may contribute to an understanding of the pathophysiological link between risk factors and atherothrombotic disease.

The correlations between each risk factor and PDNO release in this study were relatively weak. A possible reason is that the present study included a relatively small number of subjects compared with epidemiological trials. Hence, it is possible that a significant independent correlation between PDNO release and other risk factors, such as hypercholesterolemia, diabetes mellitus, or family history, would be shown in a larger sample size. In this study, the $r^2$ value for the multiple stepwise regression model of PDNO release with all independent variables was 0.51, implying that 51% of the variability of PDNO release could be explained by the risk factors. Finally, we were unable to determine with our method whether coronary risk factors impair the release of PDNO or increase the inactivation of PDNO released from platelets.

In conclusion, the present study demonstrates that coronary risk factors are associated with an impairment of PDNO release in human platelets.

Acknowledgments

This work was supported in part by a Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture.
of Japan, Tokyo, and by a research grant from the Kimura Memorial Heart Foundation, Kurume, Japan.

References
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Hypertension. 2000;35:904-907
doi: 10.1161/01.HYP.35.4.904

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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