Increased Expression of P-Selectin on Platelets Is a Risk Factor for Silent Cerebral Infarction in Patients With Atrial Fibrillation

Role of Nitric Oxide

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Background—Platelet activation and decreased levels of nitrite and nitrate (NOx), stable end products of nitric oxide (NO), are reported in patients with atrial fibrillation (AF). We examined the time-course changes in plasma NOx levels and the expression of P-selectin on platelets after the onset of AF in a canine model and determined whether these parameters could be risk factors for silent cerebral infarction in patients with AF.

Methods and Results—AF was induced by rapid atrial pacing in the canine model of AF. Plasma NOx levels were significantly decreased and the levels of P-selectin on platelets and of neutrophil/platelet conjugates were significantly increased after the onset of AF in this model. The in vitro experiments demonstrated that the inhibition of NO synthesis increased the expression of P-selectin on platelets. Plasma NOx levels (19.7±2.4 versus 27.5±2.8 μmol/L) were significantly lower in 25 patients with AF compared with age- (±2 years) and sex-matched control subjects. Conversely, the levels of P-selectin on platelets (7.6±0.8% versus 4.8±0.7%) and of neutrophil/platelet conjugates (14.8±0.9% versus 8.1±0.6%) were significantly higher in patients with AF. Multiple regression analysis revealed that increased P-selectin on platelets and advanced age were associated with the number of foci of silent cerebral infarction.

Conclusions—An irregular heart rate that is characteristic of AF appeared to blunt NO synthesis. The increased expression of P-selectin on platelets associated with the reduced NO levels was a risk factor for silent cerebral infarction in patients with AF. (Circulation. 1998;98:1721-1727.)

Key Words: fibrillation ■ endothelium-derived factors ■ risk factors ■ brain ■ infarction

Atrial fibrillation (AF) is associated with an increased risk of stroke and thromboembolism.1 Magnetic resonance imaging (MRI) can reveal a high prevalence of silent cerebral infarction (SCI) in patients with AF.2,3 Although the precise mechanism by which thromboemboli are increased in patients with AF has not been determined, hemostatic abnormalities4,5 and platelet activation6 may be contributing factors. These abnormalities are found in patients with AF even in the absence of organic valvular changes,3,6 suggesting that an irregular heart rate and the resulting turbulent flow in the systemic vessels may cause a prothrombotic state. Nitric oxide (NO), an antithrombotic product of the endothelial cells, inhibits platelet activation and prevents platelet and leukocyte adhesion to the endothelium.7,8 Although endothelial NO synthase activity is stimulated by shear stress,9 its activity is quite different under flow conditions: NO synthase activity under turbulent flow conditions, which are characteristic of AF, is markedly depressed compared with that under laminar or periodic flow conditions.10,11 We recently demonstrated that indicators of plasma NO are decreased in patients with AF associated with hemostatic abnormalities and platelet activation.12

P-selectin, an important adhesion molecule found in the secretory granules of platelets and the Weibel-Palade bodies of endothelial cells, is mobilized to the plasma membrane on activation.13 P-selectin mediates neutrophil/platelet interactions as well as neutrophil/endothelial cell interactions.13 Because the development of thrombi in vivo may involve platelet activation and the formation of neutrophil/platelet conjugates,14 P-selectin expressed on platelets may be important in the formation of thromboemboli. Recently, the reduced bioavailability of NO has been suggested to cause thrombotic disorders associated with the increased expression of P-selectin on platelets in humans.15 Therefore, we hypoth-
esized that the increased expression of P-selectin on platelets due to reduced NO levels under turbulent flow conditions contributes to the formation of thromboemboli in patients with AF. To test this idea, we examined the time-course changes of nitrite and nitrate (NOx), the expression of P-selectin on platelets, and the levels of neutrophil/platelet conjugates in a canine model of AF. Next, we determined the effect of the inhibition of NO synthesis on the expression of P-selectin on platelets in vitro. In patients with AF, we measured plasma NOx levels, the expression of P-selectin on platelets, and the levels of neutrophil/platelet conjugates compared with those in age- and sex-matched control subjects with sinus rhythm. Finally, we evaluated the clinical and hemodynamic parameters measured in the present study as potential risk factors for SCI in patients with AF.

**Methods**

**Materials**

\(^{N^\text{O}}\text{-Nitro-l-arginine methyl ester (L-NAME), l-arginine (l-arg),}\)

paraformaldehyde, and sodium citrate were obtained from Sigma Chemical Co. The monoclonal antibody (mAb) against P-selectin (CY-1747, mouse IgG\(\text{}_\text{1}\)), also known as PB1.3) was kindly donated by Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). We have previously confirmed that CY1747 recognizes P-selectin expressed on canine platelets.\(^{16}\) The mAb against CD41 (MCA 1471, mouse IgG\(\text{}_\text{2a}\)), which is commonly and specifically expressed on the surface of canine platelets\(^{16,17}\); fluorescent isothiocyanate (FITC)-conjugated goat antibody against mouse IgG\(\text{}_\text{2a}\); FITC-conjugated sheep antibody against mouse IgG\(\text{}_\text{2a}\); mAb against human P-selectin (MCA 796 F); mAb against human CD41a (MCA 467 F); and negative isotopic mAbs were purchased from Serotec. FACS lysing solution was purchased from Becton Dickinson.

**Protocol 1: Serial Changes in Plasma NOx Levels, Expression of P-Selectin on Platelets, and Levels of Neutrophil/Platelet Conjugates in the Canine Model of AF**

Ten mongrel dogs (weight, 15 to 21 kg) were anesthetized with pentobarbital sodium (30 mg/kg), the tracheas were intubated, and the animals were ventilated with room air supplemented with oxygen. Catheters were inserted into the left carotid artery and vein, and patency was maintained with heparinized saline solution. Samples of venous blood were drawn without stasis, and coagulation was inhibited with 3.8% sodium citrate (1:9, vol/vol; pH 7.4). The chest was opened through the right fourth intercostal space, and the heart was suspended in a pericardial cradle. Two bipolar Teflon-coated stainless steel clip electrodes were inserted into the right appendage in 5 dogs (AF group). AF was induced with bursts of rapid atrial pacing (interval, 20 Hz; pulse width, 4.5 ms; 3 times diastolic threshold) as described previously with minor modifications\(^{18}\) (Figure 1). Pacing was continued to induce AF again when AF was spontaneously converted to sinus rhythm during experimental protocols. Thus, we can consider AF to be sustained during the experimental protocol. Because our preliminary experiments demonstrated that the mean heart rate in this AF model was ~200 beats/min, we performed right atrial pacing at 200 beats/min in 5 dogs (control group). The standard 6-lead surface ECG was recorded. Plasma NOx levels, the expression of P-selectin on platelets, and the levels of neutrophil/platelet conjugates were measured in blood samples obtained before and 1, 2, 3, and 6 hours after the onset of atrial pacing. Plasma NOx levels were measured with the Greiss reagent as reported previously.\(^{19}\) We evaluated the expression of P-selectin on platelets and levels of neutrophil/platelet conjugate as
reported previously. Isotopic mAbs were used to determine non-specific background fluorescence in samples prepared in parallel. Samples were analyzed on a FACScan flow cytometer with CellQuest software (Becton Dickinson). A gate was set around the platelets, and a total of 5000 platelets were counted in each sample. The percentage of platelets that were positive for P-selectin was determined by the number that had FITC fluorescence of >99% of the platelets incubated with the nonspecific antibody. The percentage of neutrophils bound to platelets was determined as described previously.16

Protocol 2: Effects of L-NAME on Expression of P-Selectin on Platelets In Vitro
Samples of venous blood were drawn from 7 healthy volunteers (5 men and 2 women; mean age, 29 years) without stasis. Coagulation was inhibited with 3.8% sodium citrate (1:9, vol/vol; pH 7.4). Platelet-rich plasma (PRP) was obtained as described. To examine the role of endogenous NO produced in platelets,17 samples were incubated with L-NAME (1 mmol/L) at 37°C for 10 minutes. In another set of experiments, PRP was first incubated with L-arginine (1 mmol/L) for 10 minutes. After this incubation, PRP was incubated with L-NAME for an additional 10 minutes. The platelets were washed twice, fixed in 1% (vol/vol) paraformaldehyde, and analyzed by flow cytometry.

Protocol 3: Plasma Levels of NOx, Expression of P-Selectin on Platelets, and Levels of Neutrophil/Platelet Conjugates in Patients With AF
Twenty-five subjects with AF (17 men and 8 women) and 25 sex- and age- (±2 years) matched nonhospitalized control subjects with sinus rhythm were evaluated. Patients with chronic sustained AF but without a history of cerebrovascular accident, transient ischemic attack, mitral stenosis, left ventricular aneurysm, or intra-atrial and intraventricular thrombi on 2-dimensional echocardiography were selected for inclusion into the study. Plasma NOx levels, the expression of P-selectin on platelets, and the levels of neutrophil/platelet conjugates were determined as described. Blood was drawn from the cubitus vein in the morning after the subjects had fasted overnight.

Protocol 4: Evaluation of SCI by MRI
Twenty-five patients with AF in protocol 3 underwent brain MRI. SCI was defined as a defect on the MRI consistent with a cerebral infarct in a neurologically normal patient. MRI was performed using a field strength of 0.5 T (GE Yokogawa Medical System, MR Vector) in the orbitomedial plane with sections that were 8 mm thick in the manner of an inversion recovery technique with a repetition time of 2500 ms, an inversion time of 400 ms, and an echo time of 17 ms to achieve T1-weighted images. A spin-echo technique (repetition time, 2500 ms; echo time, 90 ms) was used to achieve T2-weighted images. The matrix consisted of 256×256 pixels. Infarction was defined as a focal area with prolonged T1 and T2 relaxation times. These lesions were visible as low and high signal intensity areas on T1- and T2-weighted images, respectively. Hyperintense images visible only on T2 images were not counted as infarctions so as to exclude perivascular space (état crible). The number of foci of SCI in each patient were counted in a blinded fashion.

Statistical Analysis
Data are expressed as mean±SEM. The time courses of changes in parameters measured in the canine model of AF were compared by 2-way repeated measures ANOVA. The effects of inhibition of NO synthesis on the expression of P-selectin on platelets were evaluated by 1-factorial ANOVA and Bonferroni’s test. Baseline characteristics of patients with and without AF were evaluated using Student’s t test for continuous variables and the χ² test for noncontinuous variables. Multiple linear regression analysis was used to evaluate the possible clinical significance of plasma NOx levels, the expression of P-selectin on platelets, neutrophil/platelet conjugates, and clinical and hemodynamic parameters measured in the number of foci of SCI. A level of P<0.05 was accepted as statistically significant.

Results
Changes in Plasma NOx Levels, Expression of P-Selectin on Platelets, and Levels of Neutrophil/Platelet Conjugates in the Canine Model of AF
Successive electrical complexes in each of the recordings varied markedly in cycle length in a canine model of AF (Figure 1). Mean heart rate increased after the onset of AF (166±4 versus 204±5 beats/min, P<0.05). Mean aortic

Figure 2. Time course of changes in plasma NOx levels (A), expression of P-selectin on platelets (B), and levels of neutrophil/platelet conjugates (C) in AF (●) and control (○) canine model. Data are expressed as mean±SEM (n=5). *P<0.05 versus baseline, †P<0.05 versus control group.
blood pressure before the onset of cardiac pacing (1124 mm Hg) did not change during the experimental protocol. At 2 hours after the onset of AF, plasma NOx levels decreased significantly (Figure 2A). The expression of P-selectin on platelets increased significantly 3 hours after the onset of AF (Figure 2B). The levels of neutrophil/platelet conjugates (Figure 2C) increased significantly after 2 hours. Neither plasma NOx levels, the expression of P-selectin on platelets, nor the levels of neutrophil/platelet conjugates changed in the control animals paced at 200 beats/min during the experimental protocol (Figure 2A through 2C).

**Effects of the Inhibition of NO Synthesis on the Expression of P-Selectin on Platelets**

Treatment with L-NAME significantly increased the fluorescence intensity of P-selectin on human platelets in vitro, as indicated by a rightward shift pattern (Figure 3). The L-NAME–induced increase in the expression of P-selectin on human platelets was blunted by pretreatment with L-arginine (Figure 4).

**Plasma NOx Levels, Expression of P-Selectin on Platelets, and Levels of Neutrophil/Platelet Conjugates in Patients With AF**

Patients with AF and the control subjects with sinus rhythm showed no significant difference in the incidence of such clinical characteristics as hypertension, diabetes, and hyperlipidemia, except for the incidence of ischemic heart disease, which was higher in the patients with sinus rhythm (Table 1). The incidence of patients with AF who were taking aspirin was higher than that of control subjects with sinus rhythm (Table 1). The incident of subjects who were taking drugs other than aspirin did not differ between the two groups. Patients with AF did not differ from control subjects with sinus rhythm with respect to hemodynamic or echocardiographic parameters that were evaluated except for the diameter of left atrium, which was larger in the patients with AF (Table 2).

Plasma NOx levels in patients with AF were lower ($P<0.05$) than those in age- and sex-matched subjects with sinus rhythm (19.7±2.4 versus 27.5±2.8 μmol/L) (Figure 5). The expression of P-selectin on platelets (7.6±0.8% versus 4.8±0.7%) and the levels of neutrophil/platelet conjugates (14.8±0.9% versus 8.1±0.6%) were greater ($P<0.05$) in subjects with AF than in subjects with sinus rhythm (Figure 5).

**Evaluation of SCI in Patients With AF**

Although none of the patients with AF in the present study had a history of cerebrovascular accident, MRI revealed the

<table>
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<tr>
<th>TABLE 1. Clinical Characteristics of Patients With Atrial Fibrillation and Those With Sinus Rhythm</th>
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<tr>
<td>Characteristic</td>
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<tr>
<td>Age, y</td>
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<td>Hypertension, %</td>
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<td>Calcium channel blocker</td>
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Values are mean±SEM.
high prevalence of SCI (92%) and numerous cerebral infarcted foci (17±4 per person; Table 3). Multiple regression analysis revealed that advanced age and increased expression of P-selectin on platelets were significantly (P<0.05) associated with the numbers of foci of SCI in patients with AF. Plasma NOx levels, the levels of neutrophil/platelet conjugates, and the remainder of the clinical and hemodynamic characteristics in Tables 1 and 2 were not significantly associated with the numbers of foci of SCI.

**Discussion**

**Plasma NOx Levels and P-Selectin on Platelets in the Canine Model of AF**

The results of the present study demonstrate that the irregular heart rate characteristic of AF can induce a decrease in plasma NOx levels. Because NO rapidly decomposes in biological solutions to form NOx,22 decreased plasma NOx levels may reflect a decrease in the plasma levels of NO in vivo. Although the precise mechanism by which plasma NOx levels were decreased in AF has not been elucidated, the decrease in NO synthase activity10,11 and damaged endothelial-
tion of P-selectin on platelets might play an important role in the thrombotic disorders in patients with AF. There are several possible mechanisms by which the expression of P-selectin on the platelets induces the formation of thromboemboli. Activation of platelets may lead to their adherence to endothelial cells, causing leukocytes within the thrombi via the P-selectin expressed on platelets. Another possibility is that the adhesion of platelets to neutrophils activates those cells. Activated leukocytes release vasoactive substances as well as reactive oxygen metabolites that may influence the process of the formation of thromboemboli and induce the vasoconstriction, thereby obstructing the small vessels. A third possibility is that conjugates of neutrophils and platelets become entrapped in the small vessels. The size of the conjugates increases as a result of homotypical leukocytes and may become sufficiently large to obstruct the capillaries.

In the present study, 52% of the subjects with AF were taking aspirin, which may have affected platelet activation. Although aspirin inhibits the release of α-granules and dense granules from the activated platelets, it does not affect the expression of P-selectin on activated platelets. Thus, aspirin appeared to have a minimal effect on the levels of the expression of P-selectin on platelets in the present study.

SCl in Patients With AF

A high incidence of SCI is reported in patients with AF. In the present study, we carefully identified the foci of SCI using both T1 and T2 images to differentiate them from the perivascular space, and we revealed the presence of SCI in >90% patients with AF. Advanced age, hypertension, diabetes mellitus, smoking, hypercholesterolemia, and cardiac diseases are previously reported to be potential risk factors of the presence of SCI. Although the population of the patients with AF was very small in the present study, multiple regression analysis indicated that advanced age and increased expression of P-selectin on platelets were significantly associated with the number of foci of SCI. This report is the first to demonstrate that the adhesion molecule on platelets that plays an important role in the formation of thrombi is a potential risk factor for SCI. Further investigation will be needed to clarify whether the increased expression of P-selectin on platelets is a potential predictor of SCI, stroke, or both in subjects with sinus rhythm and AF.

Recently, Kario et al demonstrated that endothelial cell damage is a potential risk factor for SCI in elderly Japanese with sinus rhythm, supporting the idea that reduced NO production due to endothelial cell damage might be involved in SCI in these subjects. Although we found that plasma NOX levels were decreased in both patients with AF and the canine model of AF, there was no significant relationship between plasma NOX levels and the number of foci of SCI. Further investigation is needed to clarify the involvement of NO in the pathophysiology of SCI.

In conclusion, the results of the present study suggest that the increased expression of P-selectin on platelets associated with the reduced NO levels was a potential risk factor for SCI in patients with AF. Therapy that increases NO production or that targets the P-selectin on platelets may provide a new strategy for preventing thromboembolism in patients with AF.

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