Stable Angina and Acute Coronary Syndromes Are Associated With Nitric Oxide Resistance in Platelets

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OBJECTIVES
The study examined possible clinical determinants of platelet resistance to nitric oxide (NO) donors in patients with stable angina pectoris (SAP) and acute coronary syndromes (ACS), relative to nonischemic patients and normal subjects.

BACKGROUND
We have shown previously that platelets from patients with SAP are resistant to the antiaggregating effects of nitroglycerin (NTG) and sodium nitroprusside (SNP).

METHODS
Extent of adenosine diphosphate (1 µmol/liter)-induced platelet aggregation (impedance aggregometry in whole blood) and inhibition of aggregation by NTG (100 µmol/liter) and SNP (10 µmol/liter) were compared in normal subjects (n = 43), nonischemic patients (those with chest pain but no fixed coronary disease, n = 35) and patients with SAP (n = 82) or ACS (n = 153). Association of NO resistance with coronary risk factors, coronary artery disease (CAD), intensity of angina and current medication was examined by univariate and multivariate analyses.

RESULTS
In patients with SAP and ACS as distinct from nonischemic patients and normal subjects, platelet aggregability was increased (both p < 0.01), and inhibition of aggregation by NTG and SNP was decreased (both p < 0.01). Multivariate analysis revealed that NO resistance occurred significantly more frequently with ACS than with SAP (odds ratio [OR] 2.3:1), and was less common among patients treated with perhexiline (OR 0.3:1) or statins (OR 0.45:1). Therapy with other antianginal drugs, extent of CAD, intensity of angina and coronary risk factors were not associated with variability in platelet responsiveness to NO donor.

CONCLUSIONS
Patients with symptomatic ischemic heart disease, especially ACS, exhibit increased platelet aggregability and decreased platelet responsiveness to the antiaggregatory effects of NO donors. The extent of NO resistance in platelets is not correlated with coronary risk factors. Pharmacotherapy with perhexiline and/or statins may improve platelet responsiveness to NO.

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angiotensin-converting enzyme (ACE) inhibitors and lipid-lowering agents (for review see Reference 17).

Given the pathophysiological role of incremental oxidative stress in NO resistance, the current study was undertaken to investigate whether the extent of the platelet NO resistance in patients presenting with chest pain: 1) depends on the underlying diagnosis (nonischemic chest pain, SAP or acute coronary syndromes [ACS]) and extent of fixed coronary artery disease, and 2) is associated with coronary risk factors and pharmacotherapy. The inhibitory effects of NTG and sodium nitroprusside (SNP) on adenosine diphosphate-induced platelet aggregation were examined in blood samples obtained from normal subjects, nonischemic patients with normal coronary arteries, patients with stable angina and patients with ACS in order to identify potential clinical correlates of NO resistance at the level of platelet aggregation.

**METHODS**

**Patients/normal subjects.** Patient characteristics are shown in Table 1. Patients with SAP (aged 39 to 76 years, n = 82) were evaluated at the time of diagnostic cardiac catheterization and coronary angiography. In all cases at least one hemodynamically significant (≥50%) stenosis was present in a major coronary artery. Patients with ACS (aged 37 to 98 years, n = 153) were admitted for treatment of prolonged chest pain occurring at rest and were studied during the first hour after admission; eventual diagnosis was unstable angina pectoris (n = 100) or non-Q-wave myocardial infarction (n = 53). Nonischemic patients (aged 35 to 77 years, n = 35) presented with chest pain but were found to have angiographically normal coronary arteries at diagnostic cardiac catheterization. In all patients a background medication profile was recorded at recruitment. No patient was receiving ADP receptor or glycoprotein IIb/IIIa receptor antagonists. A cohort of healthy volunteers (n = 43; 23 men and 20 women aged 23 to 76 years, mean 46 years) not taking any medication affecting platelet aggregation was also studied. In all cases, blood samples were collected for assessment of platelet aggregation. The ischemic patients were compared with nonischemic patients, who were of similar ages, and with apparently healthy volunteers. Numbers of subjects utilized in individual experiments are indicated in the Results section. The protocol was approved by the Ethics of Research Committee of The Queen Elizabeth Hospital; informed consent was obtained prior to study entry.

**Blood sampling and platelet aggregation studies.** Blood samples from patients undergoing cardiac catheterization were collected during the procedure via a femoral arterial sheath, and from other patients and normal volunteers by venesection from an antecubital vein. We have shown previously (10) that there is no arteriovenous difference in platelet function. Blood was collected in plastic tubes containing 1:10 volume of acid citrate anticoagulant (two parts of 0.1 mol/liter citric acid to three parts of 0.1 mol/liter trisodium citrate); acidified citrate was utilized to minimize deterioration of platelet function during experiments (18).

Aggregation in whole blood was examined utilizing a dual-channel impedance aggregometer (Model 560, Chrono-Log, Haverstown, Pennsylvania) as described pre-

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Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NIP (n = 35)</th>
<th>SAP (n = 82)</th>
<th>ACS (n = 153)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women, n</td>
<td>17/18</td>
<td>57/25</td>
<td>95/58</td>
</tr>
<tr>
<td>Age (mean ± SD), yrs</td>
<td>54 ± 12</td>
<td>64 ± 10</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>1 (3)</td>
<td>25 (30)</td>
<td>40 (26)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>9 (25)</td>
<td>43 (52)</td>
<td>83 (54)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>5 (14)</td>
<td>42 (51)</td>
<td>73 (48)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>5 (14)</td>
<td>10 (12)</td>
<td>34 (22)</td>
</tr>
<tr>
<td>Drugs used</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>10 (29)</td>
<td>69 (84)</td>
<td>111 (73)</td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>6 (17)</td>
<td>47 (57)</td>
<td>112 (73)</td>
</tr>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>4 (11)</td>
<td>21 (26)</td>
<td>35 (23)</td>
</tr>
<tr>
<td>SH-donors (e.g., N-AC), n (%)</td>
<td>0 (0)</td>
<td>10 (12)</td>
<td>30 (20)</td>
</tr>
<tr>
<td>Perhexiline, n (%)</td>
<td>1 (3)</td>
<td>12 (15)</td>
<td>19 (12)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>5 (14)</td>
<td>29 (35)</td>
<td>41 (27)</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-antagonists, n (%)</td>
<td>14 (40)</td>
<td>48 (59)</td>
<td>90 (59)</td>
</tr>
<tr>
<td>Beta-adrenoceptor antagonists, n (%)</td>
<td>2 (6)</td>
<td>20 (24)</td>
<td>25 (16)</td>
</tr>
</tbody>
</table>

ACE = angiotensin-converting enzyme; ACS = acute coronary syndromes; N-AC = N-acetylcysteine; NIP = nonischemic patients; SAP = stable angina pectoris; SH = sulphydryl.
Advanced age (70 years) was also treated as a risk factor and, therefore, all comparisons of platelet responses between normal subjects and different groups of patients were made utilizing analysis of variance (ANOVA) for categorical variables. Possible interactions among risk factors, therapies and platelet responsiveness to SNP and NTG were examined by stepwise multiple regression analysis (multivariate analysis). For the purpose of this analysis, NO resistance was categorized as hyporesponsiveness of platelets to SNP to an extent of 2 SD below the mean response documented in normal subjects. Statistically significant difference was limited to p < 0.05. Results are expressed as mean ± SEM unless otherwise stated.

RESULTS

Patient characteristics. Characteristics of the SAP (n = 82), ACS (n = 153) and nonischemic patient (n = 35) groups are summarized in Table 1. Of the coronary risk factors examined (male gender, age >70 years, diabetes mellitus, systemic hypertension, hypercholesterolemia and smoking), the mean number of risk factors was 2.71 ± 0.99 (SD) in the SAP group and 2.78 ± 0.89 (SD) in the ACS group. Almost all patients with either SAP or ACS were on multiple drug therapy. Numbers of cases sufficient for multivariate analysis were present in respect to therapy with aspirin, nitrates, sulphydryl donors (N-acetylcysteine or captopril), ACE inhibitors, HMG-CoA reductase inhibitors (“statins”), Ca++-antagonists, beta-adrenoceptor antagonists and perhexiline for both SAP and ACS groups. The group of nonischemic patients differed considerably from SAP or ACS groups regarding age, numbers of coronary risk factors and extent of pharmacotherapy.

ADP-induced platelet aggregation. We examined platelet responsiveness to ADP in blood samples obtained from normal subjects, nonischemic patients with normal coronary arteries, patients with SAP and with ACS. Platelet aggregation studies usually exclude individuals taking aspirin because of the suppression of thromboxane A2 generation by aspirin. However, as the majority of patients with ischemic heart disease normally receive aspirin, we included such patients in the current study. We used a relatively low concentration of ADP, 1 μmol/liter, which does not cause a release of thromboxane A2 (18). In accordance with our previous study (16), no statistically significant differences were seen in platelet responses to this concentration of ADP between patients receiving and not receiving aspirin (data not shown). Therefore, data from these patient groups were pooled; results are summarized in Table 2. Platelet aggregability in response to ADP was greater in women than in men (ANOVA: p < 0.01). With both men and women, platelets from ischemic patients were more aggregable than were those from normal subjects and from nonischemic patients (ANOVA: p < 0.01). No difference existed in aggregability between normal subjects and nonischemic patients. There was a trend toward greater aggregability in patients with ACS than in those with stable angina;

Figure 1. Representative tracings for inhibition of ADP (1 μmol/liter)-induced aggregation by nitroglycerin (NTG) (100 μmol/liter) and sodium nitroprusside (SNP) (10 μmol/liter) in a whole blood sample obtained from a normal male subject.

Data analysis. CATEGORIZATION OF CORONARY RISK FACTORS. Plasma cholesterol level >5 mmol/liter was considered as a risk factor. Cigarette smokers were defined as subjects who were current smokers. Diabetes mellitus was diagnosed according to the criteria of the World Health Organization. Hypertension was defined as previous documentation of systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg. Male gender was considered as a risk factor and, therefore, all comparisons of platelet ADP responses were stratified according to subject gender. Advanced age (≥70 years) was also treated as a potential risk factor.

STATISTICS. Comparisons of platelet responses between normal subjects and different groups of patients were made utilizing analysis of variance (ANOVA) followed by the two-sided Dunnett test (for multiple comparisons) or the Student nonpaired t test as appropriate. Univariate analysis of the effects of each potential coronary risk factor and therapy on NO responsiveness was performed with one-way ANOVA for categorical variables. Possible interactions among risk factors, therapies and platelet responsiveness to SNP and NTG were examined by stepwise multiple regression analysis (multivariate analysis). For the purpose of this analysis, NO resistance was categorized as hyporesponsiveness of platelets to SNP to an extent of 2 SD below the mean response documented in normal subjects. Statistically significant difference was limited to p < 0.05. Results are expressed as mean ± SEM unless otherwise stated.
however, these differences did not reach statistical significance.

**Inhibition of platelet aggregation by NTG and SNP.** Data from the three groups of patients examined and from normal subjects are shown in Figure 2. Inhibition of aggregation by both NTG and SNP did not vary significantly between normal subjects and nonischemic patients. Mean inhibition of aggregation by SNP in platelets from normal subjects was $66 \pm 19$ (SD)%; hence, responses of <28% inhibition, corresponding to >2 SD below normal responsiveness, were treated in multivariate analysis as NO resistance (see the following text). Responses to both NTG and SNP were markedly diminished in patients with SAP and ACS (ANOVA: $p < 0.001$ vs. normals in both groups of patients vs. normals).

**Univariate Analyses.** Determinants of interindividual variability in platelet responsiveness to NO were explored in patients with SAP and with ACS. In view of the similarity between responses to NTG and SNP in the various patient groups, only responses to SNP, a more direct NO donor than NTG (19), were utilized for univariate and multivariate analyses. Results of univariate analysis are presented in Figure 3. The majority of coronary risk factors and forms of pharmacotherapy were not associated with significant variability in SNP responses. However, statin therapy was associated with significantly increased responses ($p = 0.02$), as was therapy with perhexiline ($p = 0.01$). Surprisingly, hypercholesterolemia ($p = 0.04$) was also associated with increased response.

The results of additional analyses did not show any significant correlation (ANOVA: NS) between number of risk factors and SNP response. In patients with SAP, no association was seen between severity of angina (assessed according to the Canadian Cardiovascular Society classification of angina) and NO resistance (ANOVA: NS). Among patients with ACS, no significant difference existed in platelet responsiveness to NO donor between patients with and without myocardial infarction (ANOVA: NS). In patients with SAP, increasing extent of fixed coronary artery disease was associated with a nonsignificant (ANOVA: $p = 0.08$) trend toward decreased platelet responsiveness.

**Multivariate Analyses.** Results of multivariate analysis of factors potentially associated with the presence/absence of NO resistance are summarized in Table 3. None of the

### Table 2. Platelet Aggregation (Ohms) in Response to $1 \mu$mol/liter ADP in Whole Blood From Normal Subjects, Nonischemic Patients (NIP), Patients With Stable Angina Pectoris (SAP) and Patients With Acute Coronary Syndromes (ACS)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Normals</th>
<th>NIP</th>
<th>SAP</th>
<th>ACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>7.7 ± 0.7 (23)</td>
<td>7.3 ± 0.8 (17)</td>
<td>9.4 ± 0.5 (57)*</td>
<td>10.8 ± 0.7 (95)**</td>
</tr>
<tr>
<td>Women</td>
<td>9.9 ± 0.6 (20)</td>
<td>9.5 ± 0.6 (18)</td>
<td>13.0 ± 0.8 (25)*</td>
<td>13.7 ± 0.6 (58)**</td>
</tr>
</tbody>
</table>

Number of subjects indicated in parentheses. *$p < 0.05$ and **$p < 0.01$ vs. gender-matched normals.

![Image](file://C:/Users/username/Documents/image.jpg)
coronary risk factors considered were significant correlates of response. Both perhexiline and statin therapy remained as direct correlates of platelet responsiveness to NO, as with univariate analysis; this suggests that the apparently direct association of hypercholesterolemia with NO responsiveness on univariate analysis reflected the high proportion (59%) of such patients receiving statins. Importantly, although mean responses to SNP were not significantly different in SAP and ACS patients (Fig. 2), the presence of ACS (as distinct from SAP) emerged as a strong correlate of platelet resistance to NO on multivariate analysis (Table 3).

DISCUSSION

The results of this work provide incremental evidence linking the phenomenon of NO resistance in platelets with the presence of symptomatic myocardial ischemia: patients with SAP or ACS manifest platelet resistance to NO, as distinct from nonischemic patients, those with chest pain but normal coronary arteries (Fig. 2). Furthermore, the diagnosis of ACS, as distinct from SAP, was a determinant of a worse platelet responsiveness to SNP on multivariate analysis, with an odds ratio (OR) of 2.3:1 (Table 3). The presence of NO resistance was independent of conventional coronary risk factors and the majority of forms of pharmacotherapy (Fig. 3). However, on both univariate and multivariate analyses, both statin therapy and perhexiline therapy were associated with decreased incidence of platelet resistance to NO.

Ischemia and NO resistance. Although occurring in the presence of hyperaggregability to ADP (Table 2), NO resistance is not a consequence of increased platelet aggregation; its biochemical correlate, impaired formation of cGMP in platelets, occurs in the absence of ADP and is proportional to impairment of the antiaggregatory effects of SNP (15). Our results suggest that the extent of symptomatic ischemia may contribute to impairment of SNP response. One possible mechanism of a correlation between active ischemia and platelet resistance to NO would be incremental oxidative stress, engendered at least in part in association with activation of systemic inflammation, in particular in patients with ACS (20), as this may lead to inactivation of platelet guanylate cyclase (21) and accelerated clearance of NO by $O_2^-$ (22), both of which are implicated as probable causes of NO resistance (16). In view of these observations, it may have been expected that factors modifying oxidative stress (presence of SAP and ACS) might affect responses to NO. The results of comparisons between ACS or SAP patients and nonischemic patients are consistent with this supposition. Although the presence (vs. absence) of creatine kinase elevation was not identified as a

Figure 3. Univariate analyses: interactions of coronary risk factors and pharmacotherapy with inhibition of adenosine diphosphate-induced platelet aggregation by sodium nitroprusside (10 μmol/liter) in whole blood samples obtained from patients with stable angina pectoris and acute coronary syndromes. ACE inh = angiotensin-converting enzyme inhibitors; ASA = aspirin; CaA = calcium antagonists; H. Chol = high cholesterol; HT = hypertension; Pex = perhexiline; SH = sulphhydril. *p < 0.05. **p < 0.01.

Table 3. Multivariate Analysis: Significant Correlates of Impaired (<28%) Platelet Response to Sodium Nitroprusside (10 μmol/liter) in Blood Samples From Patients With Stable Angina Pectoris (n = 82) or Acute Coronary Syndromes (n = 153)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute coronary syndromes</td>
<td>2.29</td>
<td>1.18, 4.46</td>
<td>0.012</td>
</tr>
<tr>
<td>Perhexiline therapy</td>
<td>0.31</td>
<td>0.11, 0.88</td>
<td>0.018</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>0.45</td>
<td>0.22, 0.90</td>
<td>0.021</td>
</tr>
</tbody>
</table>

The following parameters were not significantly correlated with platelet hyporesponsiveness to sodium nitroprusside: male gender, age >70 years, diabetes mellitus, systemic hypertension, hypercholesterolemia, smoking, therapy with aspirin, nitrates, sulphhydril donors (N-acetylcysteine or captopril), angiotensin-converting enzyme inhibitors, Ca++ antagonists, and beta-adrenoceptor antagonists.
determinant of NO resistance in the current study, it is possible that if larger (Q-wave) infarcts were included, incremental inflammatory activation of neutrophils and greater redox stress might have resulted in further impairment of NTG and SNP responses. Similarly, it might also have been expected that conventional coronary risk factors would be correlated, both individually and cumulatively, with impaired responsiveness to NO. However, in a recent study in isolated human internal mammary arteries, only hyperlipidemia emerged as a significant correlate of vascular superoxide production (23). Furthermore, our results are consistent with a recent study (7) that documents no association between coronary risk factors and responsiveness to NO donors in the coronary circulation.

**Impact of therapy.** Platelet resistance to NO should be distinguished from the phenomenon of acquired nitrate tolerance, a nitrate-selective attenuation of antiaggregatory responses that does not affect response to SNP (24). In the current study, concurrent therapy with prophylactic nitrates was not associated with abnormal platelet responsiveness to SNP or to NTG. Although administration of NTG may inhibit ex vivo platelet aggregation, as we have previously reported (10), this occurs shortly after NTG intake, in experiments designed to minimize the impact of a rapid NTG catabolism (25).

Multivariate analysis revealed (as was suggested on the basis of univariate analysis) that statin therapy was associated with augmentation of platelet responsiveness to SNP; this has not previously been documented. As hypercholesterolemia was not a correlate for platelet resistance to NO, this finding cannot purely represent the cholesterol-lowering effect of these agents. A potential mechanism would be limitation of oxidative stress by statin therapy (26). Furthermore, our finding may provide a theoretical basis for potential beneficial effects of statins in unstable angina (27).

Therapy with the potent prophylactic antiangiinal agent (28,29) perhexiline was associated with augmented platelet responsiveness to SNP on both univariate and multivariate analyses, with an OR for elimination of nitrate resistance of 3.2:1. The mechanism of this change might be amelioration of ischemia (and hence of oxidative stress). A prospective study has proved that perhexiline therapy normalizes platelet responsiveness to NO donors in both SAP and ACS (30).

The lack of observed correlation between ACE inhibitor therapy and platelet response to NO may possibly be due to emergence of such effects only at high doses of ACE inhibitors (31). It is also important that changes in platelet responsiveness to NO may not necessarily parallel those of vascular smooth muscle, despite similarities between our findings and those of Zeiher’s group (7).

Platelet hyperaggregability (Table 2) combined with hyporesponsiveness to exogenous NO donors in patients with angina (Fig. 2) reflects an impaired physiological response to endogenous NO (endothelium-derived relaxing factor), and as such it represents a potential contribution to the increased risk of ischemic events in such patients. Impairment in responsiveness to NO in ischemic patients implies a potential problem, namely that those in need of nitrate therapy may be least likely to respond.

**Conclusions.** The results of the current study shed new light on the importance of platelet responsiveness to NO in angina pectoris. First, it appears that platelet resistance to NO is a characteristic of patients with severe SAP or ACS. It is likely that this may contribute both to the pathogenesis of ischemia in these circumstances and also to the relatively poor responsiveness to organic nitrates in many of these patients. Furthermore, impaired responsiveness to NO is likely to contribute, together with the decreased NO generation/release from platelets (32), to platelet hyperaggregability in patients with angina pectoris. The finding in the current study that therapy with statins and with perhexiline is associated with a lower incidence of platelet resistance to NO should provide an additional impetus toward investigations of both of these types of agents in the prophylaxis and treatment of acute and chronic myocardial ischemia.

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