Association Between Increased Platelet P-Selectin Expression and Obesity in Patients With Type 2 Diabetes

A BARI 2D (Bypass Angioplasty Revascularization Investigation 2 Diabetes) substudy

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OBJECTIVE — To determine whether obesity increases platelet reactivity and thrombin activity in patients with type 2 diabetes plus stable coronary artery disease.

RESEARCH DESIGN AND METHODS — We assessed platelet reactivity and markers of thrombin generation and activity in 193 patients from nine clinical sites of the Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D). Blood taken at the time of enrollment was used for assay of the concentration of prothrombin fragment 1.2 (PT1.2, released when prothrombin is activated) and fibrinopeptide A (FPA, released when fibrinogen is cleaved). Platelet activation was identified with the use of flow cytometry in response to 0, 0.2, and 1 μmol/l adenosine diphosphate (ADP).

RESULTS — Concentrations of FPA, PT1.2, and platelet activation in the absence of agonist were low. Greater BMI was associated with higher platelet reactivity in response to 1 μmol/l ADP as assessed by surface expression of P-selectin (r = 0.29, P < 0.0001) but not reflected by the binding of fibrinogen to activated glycoprotein IIb-IIIa. BMI was not associated with concentrations of FPA or PT1.2. Platelet reactivity correlated negatively with A1C (P < 0.04), was not related to the concentration of triglycerides in blood, and did not correlate with the concentration of C-reactive peptide.

CONCLUSIONS — Among patients enrolled in this substudy of BARI 2D, a greater BMI was associated with higher platelet reactivity at the time of enrollment. Our results suggest that obesity and insulin resistance that accompanies obesity may influence platelet reactivity in patients with type 2 diabetes.
agulated with 32 μg/ml corn trypsin inhibitor (CTI; Enzyme Research, South Bend, IN). CTI is a specific inhibitor of Factor XIa without effect on other coagulation factors (11) and was used as the anticoagulant because we have shown that the activation of platelets is altered by conventional anticoagulants such as citrate (12).

Activation of platelets was identified with the use of flow cytometry by the binding to platelets of fluorescein isothiocyanate–labeled fibrinogen (that binds to the activated conformation of glycoprotein IIb-IIIa) and phycoerythrin-labeled anti-CD62 that binds to P-selectin on the surface of activated platelets (Becton-Dickinson) as previously described (5,13,14). Platelets were identified based on size and binding of a peridinin chlorophyll protein (PerCP)-labeled anti-CD61 (Becton Dickinson), which binds to glycoprotein IIa regardless of activation and does not interfere with the binding of fibrinogen.

To quantify nonspecific association of proteins with platelets and to define a threshold above which activation-dependent association occurs, control samples containing phycoerythrin-conjugated nonimmune mouse IgG and fluorescein isothiocyanate–conjugated albumin were assayed in samples from each subject. Assays were performed in duplicate. Activation of platelets was reported as the percentage of platelets that bound fluorescein isothiocyanate–labeled fibrinogen or phycoerythrin–anti-CD62. This measure of platelet activation correlates with subsequent cardiac risk (13) and with platelet activation reported as the mean fluorescence intensity (14).

**FPA and PT1.2**

Blood to be analyzed for PT1.2 and FPA was added immediately to SCAT-1 tubes (Hematologic Technologies, Essex, VT). These tubes prevent protease activity and activation of coagulation factors. Aliquots of plasma were stored at −80°C until assay.

Concentrations of PT1.2 and FPA were determined with the use of commercial enzyme-linked immunosorbent assay (ELISA) kits (Dade Behring [Marburg, Germany] for PT1.2 and Vitro Chemie [Toernooiveld, the Netherlands] for FPA).

**Biochemical parameters**

Concentrations of C-reactive protein (CRP) were determined with the use of a high-sensitivity colorimetric competitive ELISA as previously described (15). Concentrations of insulin were determined by ELISA (ALPCO, Salem, NH). Concentrations of fibrinogen were determined by nephelometry (Siemens Healthcare Diagnostics, Deerfield, IL).

Fasting lipid profile and A1C assays were performed by the Biochemistry Core Laboratory at the University of Minnesota, Minneapolis. Triglycerides were analyzed enzymatically. A1C concentrations were analyzed by high-performance liquid chromatography. For those patients for whom the core lab measures were unavailable, an estimate based on sitespecific regression of the clinical site measure was used. This estimation was used for 2.5% of the triglycerides samples and 0.5% for A1C samples.

**Analysis of data**

We report results from patients in which at least 80% of significant baseline data points were available for analysis (n = 201). Patients treated with either ticlopidine or clopidogrel were excluded from this analysis (n = 8) because a primary measure was ADP-induced activation of platelets. This study group (n = 193) enabled us to identify for 1 SD change in the independent variable a regression coefficient of 0.20 for 1 SD change in a dependent variable with a power of 0.77.

Because platelet reactivity results and those for markers of thrombosis were not available, an estimate based on site-specific regression of the clinical site measure was used. This estimation was used for 2.5% of the triglycerides samples and 0.5% for A1C samples.

### Table 1—Demographic and clinical characteristics

| Characteristic                                      | Value     |
|----------------------------------------------------|-----------|
| Female (%)                                         | 31.8      |
| Age at study entry (years)                         | 62.5 ± 9.1|
| Duration of diabetes (years)                       | 10 ± 8.7  |
| Hypertension requiring treatment (%)               | 84.0      |
| History of myocardial infarction (%)               | 37.0      |
| Prior coronary artery bypass grafting (%)          | 2.0       |
| Prior percutaneous coronary intervention (%)       | 19.9      |
| Current smoker (%)                                 | 6.0       |
| BMI                                                | 30.5 ± 5.4|
| BMI categories (%)                                 |           |
| Low, <20                                           | 1.0       |
| Normal, 20 to <25                                  | 11.0      |
| Overweight, 25 to <30                              | 37.0      |
| Class 1 obesity, 30 to <35                         | 33.0      |
| Class 2 obesity, 35 to <40                         | 11.5      |
| Class 3/4 obesity, ≥40                            | 6.5       |
| Waist circumference (cm)                           | 104.8 ± 13.1|
| Metabolic syndrome (%)                             | 93.0      |
| PVD (%)                                            | 32.1      |
| A1C (%)                                            | 7.8 ± 1.7 |
| Total cholesterol (mg/dl)                          | 172 ± 41  |
| Triglycerides (mg/dl) [median (Q1–Q3)]             | 179 (108–212) |
| HDL cholesterol (mg/dl)                            | 37 ± 9    |
| LDL cholesterol (mg/dl)                            | 101 ± 34  |
| Systolic blood pressure                            | 139.3 ± 24.7|
| Diastolic blood pressure                           | 80.2 ± 14.3|
| Blood pressure >130/80 mmHg (%)                    | 62.3      |
| Heart rate (bpm)                                   | 68.3 ± 12.3|
| Baseline medications (%)                           |           |
| Aspirin                                            | 89.4      |
| ß-Blocker                                          | 72.6      |
| Calcium-channel blocker                            | 29.9      |
| ACE/angiotensin receptor blocker                   | 71.1      |
| Nitrates                                           | 40.8      |
| Statin                                             | 72.6      |
| Insulin                                            | 25.9      |
| Oral hypoglycemic                                  | 81.1      |

Data are means ± SD unless otherwise stated. n = 193. Metabolic syndrome is defined by two of the following: large waist circumference, high triglycerides, low HDL cholesterol, or high blood pressure. PVD is defined as any of ABI ≤ 0.9, carotid stent, carotid disease, carotid surgery, intermittent claudication, and non-coronary vascular surgery. IQR, interquartile range; Q1, first quartile; Q3, third quartile.
Platelet reactivity correlates with BMI in diabetes

Table 2—Biochemical markers

|                      | n  | Median | First quartile | Third quartile |
|----------------------|----|--------|----------------|---------------|
| PT 1.2 (mmol/l)      | 161| 0.84   | 0.60           | 1.26          |
| FPA (ng/ml)          | 127| 6.75   | 3.92           | 17.36         |
| Fibrinogen           | 191| 361    | 308            | 415           |
| P-selectin 0 μmol/l ADP | 180| 0.1    | 0.0            | 0.4           |
| P-selectin 0.2 μmol/l ADP | 180| 1.8    | 0.7            | 4.45          |
| P-selectin 1 μmol/l ADP | 180| 7.90   | 3.0            | 19.85         |
| Fibrinogen binding 0 μmol/l ADP | 180| 0.85   | 0.1            | 2.80          |
| Fibrinogen binding 0.2 μmol/l ADP | 180| 17.55  | 9.30           | 30.45         |
| Fibrinogen binding 1 μmol/l ADP | 180| 58.05  | 38.60          | 77.70         |
| CRP (μg/ml)          | 191| 2.05   | 0.73           | 5.06          |
| Triglycerides (mg/dl)| 193| 146    | 108            | 212           |
| A1C (%)              | 293| 7.6    | 6.3            | 8.9           |

normally distributed, Spearman's rank correlation estimates were used to evaluate relationships among the thrombosis markers and other variables of interest including BMI, A1C, lipids, and insulin. A P value of 0.05 was considered significant.

RESULTS

Patient characteristics

Clinical characteristics of patients are shown in Table 1.

Markers of thrombosis and platelet reactivity

Evidence of thrombin generation (PT1.2) and thrombin activity (FPA) was limited in these patients with stable coronary artery disease (Table 2). Similarly, evidence of platelet activation in the absence of agonist was minimal, whether assessed by the percentage of platelets that bound fibrinogen (reflecting activation of glycoprotein IIb-IIIa) or the surface expression of P-selectin (Table 2). The concentration of fibrinogen correlated positively with the percentage of platelets that bound fibrinogen with no agonist and in response to 0.2 μmol/l ADP was 0.31; between 0.2 and 1 μmol/l ADP, it was 0.64 (P < 0.0001 for both). The correlation coefficient between no agonist and 1 μmol/l ADP was 0.12 (P = 0.12).

Correlation between platelet reactivity, thrombin activity, and BMI

Greater BMI was associated with greater platelet reactivity as assessed by the surface expression of P-selectin (Fig. 2). After adjustment for age, sex, A1C, use of insulin, and duration of diabetes in a linear model of platelet reactivity, the relationship between platelet reactivity and BMI remained significant (Table 3). A similar magnitude of increase was seen in the small group (n = 19) of subjects who were not taking aspirin at the time when blood was taken (data not shown). Identification of platelet activation based on the binding of fibrinogen (activation of glycoprotein IIb-IIIa) did not correlate with BMI. The concentration of FPA and PT1.2 did not correlate with BMI. By contrast, the concentration of fibrinogen correlated with BMI (r = 0.21, P = 0.004).

Obesity has been associated with poor glycem control, hypertriglyceridemia, inflammation (reflected by an increased CRP), and insulin resistance (16,17). A1C correlated negatively with platelet reactivity in response to 1.0 μmol/l ADP (r = −0.16, P < 0.04). The effects of A1C and BMI were independent. The concentration in blood of triglycerides was not correlated with platelet reactivity. Similarly, the concentration of CRP did not correlate with platelet reactivity. The fasting concentration of insulin did not correlate with platelet reactivity (r = 0.14, P = 0.07).

CONCLUSIONS—In this substudy of BARI 2D, we assessed markers of thrombin generation and activity as well

Figure 1—Distribution of platelet activation in response to ADP. The activation of platelets induced by 0.2 μmol/l ADP and 1 μmol/l ADP was quantified with the use of flow cytometry based on the surface expression of P-selectin or the binding of fluorochrome-labeled fibrinogen. Each box plot of the distribution of the percentage of platelets activated shows the median (line), the 25th and 75th percentile (box), and the 10th and 90th percentile (error bars).
as platelet reactivity in blood from patients with type 2 diabetes and stable coronary artery disease. Activity of the coagulation cascade was limited, as was evidence of platelet activation in the absence of agonist. We assessed platelet reactivity by determining the propensity of platelets to activate in response to an agonist (0.2 and 1 μmol/l ADP). We found that greater BMI was associated with progressively greater platelet reactivity when platelet activation was assessed by the surface expression of P-selectin induced by 1 μmol/l ADP. A1C correlated negatively with this measure of platelet reactivity. Triglycerides did not correlate with platelet reactivity. Thus, our results suggest that obesity increases platelet P-selectin expression in patients with type 2 diabetes.

Aspirin was used in the majority (89%) of the subjects we studied. The relationship between obesity and platelet reactivity was of similar magnitude in patients regardless of aspirin use or nonuse. These results are consistent with the limited efficacy of aspirin in patients with diabetes (20). One mechanism that may contribute to limited efficacy of antiplatelet therapy in such patients is persistently increased platelet reactivity. Our results suggest that obesity may be a cause of persistently increased platelet reactivity in patients with diabetes and thereby contribute to a lack of efficacy of aspirin.

We did not identify an association between the concentration in blood of CRP and platelet reactivity. By contrast, evidence of platelet activation has been associated with concentrations of CRP in obese women without diabetes (21). In our study, all patients had type 2 diabetes, and platelet reactivity was assessed ex vivo. The previous study compared markers of platelet activation in vivo in obese and nonobese women without diabetes. In addition, the consistent use of statins may have decreased CRP and obscured a potential interaction (22). Thus, differences in the clinical characteristics of patients and the methods used to assess platelet reactivity may account for the lack of association in our study.

Table 3—Linear model of log (platelet surface expression of P-selectin [activation]) in response to 1 μmol/l ADP

| Coefficient from multivariable model | P       |
|-------------------------------------|---------|
| $R^2 = 0.1169$                      |         |
| BMI (per 5 units)                   | 0.27    | 0.0002 |
| Age (per 10 years)                  | 0.05    | 0.60   |
| Female sex                          | -0.15   | 0.37   |
| A1C                                 | -0.10   | 0.044  |
| Insulin use                         | 0.14    | 0.48   |
| Diabetes duration (years)           | 0.006   | 0.55   |

$n = 179$. 

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Although we did not specifically measure insulin sensitivity, obesity and increased concentrations of insulin in fasting blood have been associated with insulin resistance (16). The strong positive association that we observed between obesity and platelet reactivity is consistent with our hypothesis that insulin sensitivity influences platelet reactivity when identified based on the surface expression of P-selectin and only in response to 1 μmol/l ADP. This correlation was not apparent when activation of platelets was identified based on the binding of fibrinogen. The mechanism responsible for this difference is not apparent. However, we have previously observed that platelet activation identified based on surface expression of P-selectin is altered by associated conditions or treatments (5,23). One mechanism potentially contributing is that activation of glycoprotein Ib-IIIa occurs with a low concentration of agonist (13). Thus, the low threshold for activation of glycoprotein Ib-IIIa may limit sensitivity for detection of changes. As seen in Fig. 1, surface expression of P-selectin was limited in response to 0.2 μmol/l ADP. Thus, the discrimination of inter-individual differences is reduced when the range of platelets activated is limited. Accordingly, we postulate that the lack of statistical significance between BMI and surface expression of P-selectin in response to 0.2 μmol/l ADP reflected the limited discrimination between individuals because of the limited range of activation.

Our study does not identify the mechanism by which insulin resistance increases platelet reactivity. Previous work has associated obesity with impaired synthesis and activity of cyclic nucleotides (i.e., cyclic adenosine monophosphate and cyclic guanosine monophosphate), which are key signaling molecules involved in the activation of platelets (24).

In summary, we found that obesity is associated with greater platelet reactivity in patients with type 2 diabetes and stable coronary artery disease. Our results extend previous observations made in subjects without diabetes to subjects with diabetes and suggest that insulin resistance that is associated with obesity increases platelet reactivity that may in turn increase the risk of subsequent cardiac events in patients with type 2 diabetes.

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