

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 $\mu\text{mol/l}$ 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 μm 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.

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A pro-thrombotic state is seen in patients with diabetes and contributes to an increased rate of progression of atherosclerosis as well as to exaggerated thrombosis in response to rupture of an atherosclerotic plaque (1). Thus, diabetes is associated with more extensive vascular disease (2) and a greater burden of thrombus and associated risk of arterial occlu-

sion after rupture of an atherosclerotic plaque (3). The pro-thrombotic state reflects increased activity of platelets (4,5), increased activity of the coagulation cascade, and impaired fibrinolysis (1,6,7).

The Bypass Angioplasty Revascularization Investigation 2 Diabetes (BARI 2D) study was designed to simultaneously compare the effect on total mor-

tality of 1) revascularization combined with aggressive medical treatment as opposed to aggressive medical treatment alone with deferred revascularization as needed and 2) an insulin sensitization strategy of glycemic control as opposed to an insulin-providing strategy of glycemic control (8). A total of 2,368 patients with type 2 diabetes were enrolled at 49 clinical centers throughout North America, South America, and Europe. For this substudy of BARI 2D, 201 subjects were enrolled at nine clinical centers. We assessed platelet reactivity with the use of flow cytometry and activity of the coagulation cascade by measuring the concentration in blood of fibrinopeptide A (FPA, released during the generation of fibrin) and prothrombin fragment 1.2 (PT1.2, released during the generation of thrombin). In this study, we sought to identify clinical characteristics that influence platelet reactivity and thrombin activity in patients with diabetes and stable coronary artery disease. We hypothesized that obesity, which is associated with insulin resistance, increases platelet reactivity in patients with diabetes in a manner similar to that seen in individuals without diabetes (9,10).

RESEARCH DESIGN AND METHODS

Centers participating in BARI 2D were invited to participate in this substudy, and nine clinical centers (please see ACKNOWLEDGMENTS) agreed. All patients enrolled at each participating center were eligible to participate in this substudy. Written informed consent for participation in the substudy was obtained from each participant, and the protocol was reviewed by the institutional review board of each participating clinical center. Results were reviewed by the data and safety monitoring board of the parent BARI 2D study.

Assessment of platelet reactivity

Blood was obtained with the use of a two-syringe technique in which the first 3 ml blood was discarded. Blood (1 ml) to be analyzed for platelet reactivity was antico-

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agulated with 32 $\mu\text{g/ml}$ corn trypsin inhibitor (CTI; Enzyme Research, South Bend, IN). CTI is a specific inhibitor of Factor XIIIa 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 conformer 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 IIIa 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-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

Table 1—Demographic and clinical characteristics

Female (%)	31.8
Age at study entry (years)	62.5 \pm 9.1
Duration of diabetes (years)	10.0 \pm 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 \pm 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, \geq 40	6.5
Waist circumference (cm)	104.8 \pm 13.1
Metabolic syndrome (%)	93.0
PVD (%)	32.1
A1C (%)	7.8 \pm 1.7
Total cholesterol (mg/dl)	172 \pm 41
Triglycerides (mg/dl) [median (Q1–Q3)]	179 (108–212)
HDL cholesterol (mg/dl)	37 \pm 9
LDL cholesterol (mg/dl)	101 \pm 34
Systolic blood pressure	139.3 \pm 24.7
Diastolic blood pressure	80.2 \pm 14.3
Blood pressure >130/80 mmHg (%)	62.3
Heart rate (bpm)	68.3 \pm 12.3
Baseline medications (%)	
Aspirin	89.4
β -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 \pm 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 \leq 0.9, carotid stent, carotid disease, carotid surgery, intermittent claudication, and non-coronary vascular surgery. IQR, interquartile range; Q1, first quartile; Q3, third quartile.

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 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.

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

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 $\mu\text{mol/l}$ ADP	180	0.1	0.0	0.4
P-selectin 0.2 $\mu\text{mol/l}$ ADP	180	1.8	0.70	4.45
P-selectin 1 $\mu\text{mol/l}$ ADP	180	7.90	3.00	19.85
Fibrinogen binding 0 $\mu\text{mol/l}$ ADP	180	0.85	0.10	2.80
Fibrinogen binding 0.2 $\mu\text{mol/l}$ ADP	180	17.55	9.30	30.45
Fibrinogen binding 1 $\mu\text{mol/l}$ ADP	180	58.05	38.60	77.70
CRP ($\mu\text{g/ml}$)	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 that of PT1.2 ($r = 0.17$, $P = 0.034$) but did not correlate with the concentration of FPA ($P = 0.24$).

To assess the propensity of platelets to activate in response to a stimulus (i.e., platelet reactivity), platelets were stimulated with 0.2 and 1 $\mu\text{mol/l}$ ADP (Fig. 1). The activation of platelets identified by the surface expression of P-selectin in the absence of agonist and in the presence of 0.2 and 1 $\mu\text{mol/l}$ ADP correlated positively (the correlation coefficient between 0.2 and 1 $\mu\text{mol/l}$ ADP was 0.85, between no agonist and 0.2 $\mu\text{mol/l}$ was 0.58, and between no agonist and 1 $\mu\text{mol/l}$ ADP was 0.51; for each association, $P < 0.0001$). Correlations between the activation of glycoprotein IIb-IIIa seen with

each concentration of ADP were not as strong but were still positive. The correlation coefficient between the percentage of platelets that bound fibrinogen with no agonist and in response to 0.2 $\mu\text{mol/l}$ ADP was 0.31; between 0.2 and 1 $\mu\text{mol/l}$ ADP, it was 0.64 ($P < 0.0001$ for both). The correlation coefficient between no agonist and 1 $\mu\text{mol/l}$ 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 glycemic control, hypertriglyceridemia, inflammation (reflected by an increased CRP), and insulin resistance (16,17). A1C correlated negatively with platelet reactivity in response to 1.0 $\mu\text{mol/l}$ ADP ($r_s = -0.16$, $P < 0.04$). 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 ($r_s = 0.14$, $P = 0.07$).

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

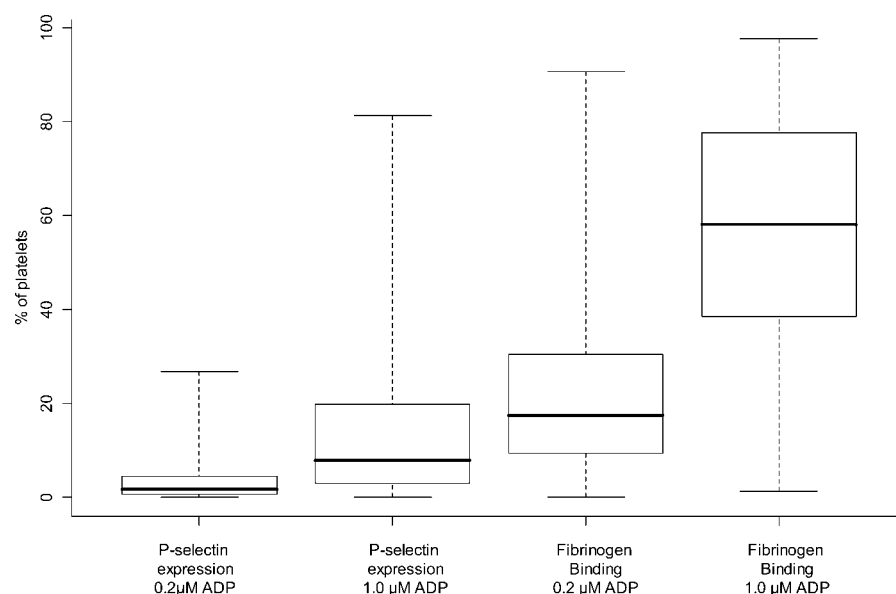


Figure 1—Distribution of platelet activation in response to ADP. The activation of platelets induced by 0.2 $\mu\text{mol/l}$ ADP and 1 $\mu\text{mol/l}$ ADP was quantified with the use of flow cytometry based on the surface expression of P-selectin or the binding of fluorochrome-labeled fibrinogen. 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).

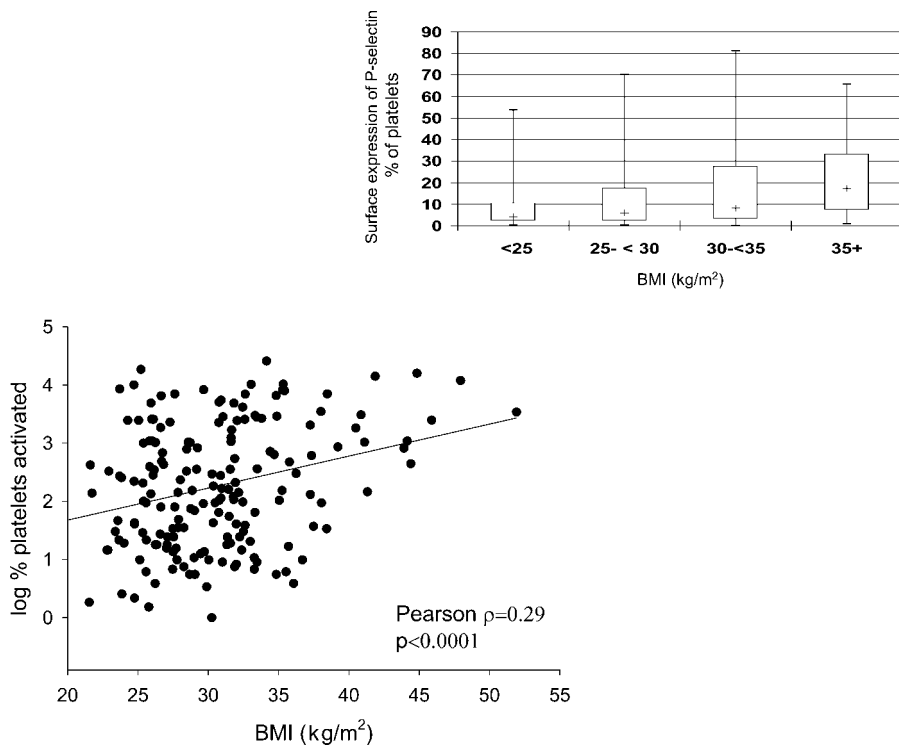


Figure 2—Correlation between the activation of platelets identified by the surface expression of P-selectin in response to 1 $\mu\text{mol/l}$ ADP and BMI in patients with diabetes. Because the activation of platelets was not normally distributed, the results were log transformed. Pearson correlation coefficient and P value are shown. The inset is a box plot showing the distribution of platelet activation in patients with a lean BMI (<25 kg/m²), overweight patients (BMI 25–30 kg/m²), obese patients (BMI 30–35 kg/m²), and morbidly obese patients (>35 kg/m²). A stepwise increment was seen with increasing BMI ($P < 0.02$).

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 $\mu\text{mol/l}$ 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 $\mu\text{mol/l}$ 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.

Increased platelet reactivity has been associated with a greater risk of subsequent cardiac events in apparently healthy subjects (18), in patients with previous myocardial infarction (19), and in patients undergoing percutaneous cor-

onary intervention (13). Our results suggest that obesity may increase further the risk of subsequent cardiovascular events in patients with type 2 diabetes by increasing platelet reactivity.

Consistent with our results, previous studies have demonstrated increased activation of platelets in obese subjects. In one study, platelet activation was identified based on measurement of a thromboxane metabolite in urine and the concentration in blood of the CD40 ligand. Both were increased in obese compared with lean women, and successful weight loss led to decreased concentrations of these markers (9). A second study quantified the concentration in blood of platelet-derived microparticles that are released during the activation of platelets. The concentration of microparticles was increased in obese patients (10). Similar to the previous study, the concentration of microparticles decreased after weight reduction (10). Accordingly, obese subjects without diabetes manifest evidence of greater activation of platelets. Our results are consistent with and extend these observations to 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 $\mu\text{mol/l}$ 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.

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 $\mu\text{mol/l}$ 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 IIb-IIIa occurs with a low concentration of agonist (13). Thus, the low threshold for activation of glycoprotein IIb-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 $\mu\text{mol/l}$ 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 $\mu\text{mol/l}$ 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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