Postprandial Platelet Activation Is Related to Postprandial Plasma Insulin Rather Than Glucose in Patients With Type 2 Diabetes

Galia Spectre,[1,2] Claes-Göran Östenson,[3] Nailin Li,[1] and Paul Hjemdahl[1]

Postprandial hyperglycemia is associated with platelet activation. We thus investigated if meal-induced platelet activation could be attenuated by meal insulin. A randomized, double-blind, crossover study was performed to compare postprandial platelet activation after premeal injections of placebo or insulin aspart (0.1 and 0.2 units/kg) in 18 patients with type 2 diabetes mellitus (T2DM). Platelet activation was assessed by flow cytometry, without and with stimulation by the thromboxane analog U46619 or ADP. Measurements were before and after premeal blood glucose standardization (to 6–7 mmol/L by insulin infusion, if needed) and at 90 min after the meal. Premeal insulin reduced postprandial hyperglycemia by 2–3 mmol/L compared with placebo. Postmeal insulin levels were doubled with placebo and further elevated with insulin injections. The standardized meal enhanced U46619-induced platelet P-selectin expression by 23% after placebo; this response was more than doubled after premeal insulin. U46619-induced fibrinogen binding was unchanged after meal intake with placebo but was markedly enhanced (by ~50–60%) after premeal insulin. Postprandial platelet activation correlated positively to postprandial insulin levels and inversely to glucose levels. Premeal insulin infusion was also associated with platelet activation. Our results suggest that postprandial insulin rather than glucose accounts for postprandial platelet activation in T2DM patients. *Diabetes* 61:2380–2384, 2012

Postprandial hyperglycemia (PPH) is thought to be an independent risk factor for cardiovascular complications in diabetes mellitus (DM) (1). PPH is associated with increased oxidative stress (2), endothelial dysfunction (3), and platelet activation (4–6). In addition, control of PPH is essential to achieve the recommended target HbA_{1c} levels (7).

Several new drugs targeting PPH have been developed (1), including the rapidly acting insulins. Insulin aspart reduces PPH and improves endothelial dysfunction (3). We have found that a carbohydrate-rich meal increases platelet activation and platelet–leukocyte conjugation stimulated by ADP (4) and, in particular, the thromboxane A_2 analog U46619 (5) in type 2 DM (T2DM). Because healthy control subjects developed neither PPH nor increased platelet reactivity after this meal (5), we hypothesized that the postprandial platelet hyperactivity was related to the hyperglycemia.

From the [1]Department of Medicine Solna, Clinical Pharmacology Unit, Karolinska University Hospital-Solna, Stockholm, Sweden; the [2]Department of Hematology, Coagulation Unit, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; and the [3]Department of Molecular Medicine and Surgery, Endocrinology & Diabetology Unit, Karolinska Institutet, Karolinska University Hospital-Solna, Stockholm, Sweden.
Corresponding author: Paul Hjemdahl, paul.hjemdahl@ki.se.

Treatment with repaglinide or glibenclamide reduced PPH only mildly and did not counteract the postprandial platelet activation (4,5).

The current study was designed to evaluate if platelet activation after a carbohydrate-rich meal is related to the PPH and thus can be attenuated by premeal insulin treatment in patients with T2DM.

RESEARCH DESIGN AND METHODS

The study was a randomized, double-blind, cross-over study comparing postprandial platelet activation after three premeal treatments: placebo (saline) or either of two doses of subcutaneous insulin aspart (Novorapid, Novo Nordisk, Bagsvaerd, Denmark), 0.1 or 0.2 units/kg body weight. The study was performed according to good clinical practice rules and was approved by the regional ethical review board and the Swedish Medical Products Agency (EudraCT No. 2006-007031-27). All patients gave written informed consent.

Patients. T2DM patients were eligible if younger than age 70 years, without known cardiovascular disease, and treated with insulin and/or metformin. HbA_{1c} levels were between 52 and 83 mmol/mol (6.9–9.7% by the Diabetes Control and Complications Trial method or 6–9% by the Mono-S method) Exclusion criteria were contraindication to insulin, acute or chronic kidney or liver diseases, or treatment with sulfonylurea, glitazones, antiplatelets, or anticoagulants.

Study design. Each patient participated in the study on three occasions separated by at least 2 weeks. Patients were instructed to abstain from food, tobacco, or nicotine on days of the experiment. No blood glucose–lowering medication was given in the mornings before experiments. The basal insulin dose was halved the evening before to minimize the risk of hypoglycemia. Patients arrived in the morning and rested in a semireclining position for 30 min, after which baseline blood samples (baseline 1 [B1]) were drawn for platelet function, glucose, and insulin levels. Premeal blood glucose levels were "standardized" to 6–7 mmol/L on every occasion by intravenous infusion of insulin aspart, if needed. A second set of blood tests was taken (baseline 2 [B2]) 15 min after the target premeal glucose level was achieved. Patients who already had glucose below 7 mmol/L at B1 rested 45 min before B2 measurements. After B2 sampling, one of the three treatments—insulin aspart at 0.1 or 0.2 units/kg body weight or placebo (in a randomized order)—was injected subcutaneously immediately before the standardized meal (54% carbohydrates, 30% fat, and 16% protein, 620 kcal) (4,5) was ingested during a 15-min period. Patients rested another 90 min before the postprandial (PP) sampling.

Blood tests. At B1, after premeal glucose titration (B2), and 90 min after the meal (PP), a complete blood cell count, triglyceride levels, and platelet activation measurements were taken. Plasma glucose (glucose oxidase technique; Hemocue Glucose 201+, Hemocue AB, Ängelholm, Sweden), insulin (radioimmunoassay [6]), and C-peptide (Human C-Peptide RIA, Millipore, Billerica, MA) were measured before and repeatedly after the meal.

Flow cytometry. Platelet P-selectin expression and fibrinogen binding were measured by whole blood flow cytometry (8,9) and are reported as percentages of positive cells in the platelet population. Platelet agonists were U46619 (thromboxane A_2 analog, 0.3 μmol/L), ADP (1 μmol/L), and a collagen-related peptide (CRP-18/i; 0.3 μg/mL), which activates platelet glycoprotein VI (GPVI) (10).

Statistics. Continuous data were compared by repeated-measures ANOVA, with Fisher post hoc testing to control for multiplicity. Differences between two treatments were analyzed by the Student paired t test after validation for normal distribution (Shapiro-Wilk test). Wilcoxon matched pairs test was used for skewed variables. The Pearson correlation coefficient was used to test independence between variables. Trend analyses were performed by means of regression analysis. Mean values and SDs are given unless otherwise specified. Analyses were done using STATISTICA 7 software (StatSoft, Tulsa, OK), and probability (P) values are given.

We estimated that 15 subjects were required to detect a 20% effect of interventions at the 5% level of significance using ADP-induced platelet P-selectin expression. When stimulated by 0.3 μmol/L U46619, the percentage of P-selectin–positive platelets increased by 9.66% (Δ value) after the meal in ref. 5. Assuming an SD of 6.63 for a 50% reduction of this Δ value, we would need 15 patients to achieve the desired statistical power ($\alpha = 0.05$; $1-\beta = 0.8$).

RESULTS

Patients. Of 27 screened patients, 18 were enrolled. Reasons for exclusion were failure to meet HbA$_{1c}$ criteria, anticoagulant treatment, thrombocytopenia, or technically suboptimal venipunctures. One patient withdrew consent after the first visit. Patient characteristics are presented in Table 1.

Glucose, insulin, and C-peptide levels. Basal fasting levels (B1) of glucose, insulin, and C-peptide were similar on the three occasions (Fig. 1, Table 2). Insulin infusion was needed in 16 patients on 43 occasions to reduce premeal glucose to 6–7 mmol/L; 2 patients needed no insulin. After the glucose standardization procedure (B2), insulin levels increased, and glucose and C-peptide levels decreased compared with B1. Premeal glucose, insulin, and C-peptide levels at B2 were very similar on the three experimental occasions (Fig. 1, Table 2).

Premeal insulin reduced postprandial plasma glucose significantly (by 2–3 mmol/L at PP) compared with placebo (Fig. 1), but the difference between doses was not significant ($P = 0.16$). Postprandial plasma insulin increased with placebo (representing endogenous insulin secretion) and was further increased after premeal injections of insulin aspart. Maximal levels of insulin were achieved 30–60 min after the meal (Fig. 1); 90 min after the meal (PP),

plasma insulin was higher after 0.2 compared with 0.1 units/kg insulin aspart ($P = 0.004$). Postprandial C-peptide levels increased after the meal, with the greatest increment after placebo (Fig. 1, Table 2).

FIG. 1. Concentrations for plasma (P) glucose (*A*), insulin (*B*), and C-peptide (*C*) are shown on the three experimental occasions. Measurements were performed before (B1) and after (B2) blood glucose standardization (by insulin infusion, if needed) to 6–7 mmol/L before the experimental treatment and meal intake. Measurements after the meal were as indicated, with the last measurement after 90 min (PP). Premeal treatments were placebo (solid lines) and insulin aspart at 0.1 units/kg (dashed lines) and at 0.2 units/kg (dotted lines). For statistics, see Table 2 and RESULTS. Mean values and SEM are shown from 18 patients.

TABLE 1
Patient characteristics

Patients	
Male	15 (83)
Female	3 (17)
Age (years)	62 (51–69)
Duration of DM (years)	10 (2–24)
Microvascular complications*	14 (78)
Hypertension†	11 (61)
Hyperlipidemia†	11 (61)
Current smokers	1 (5)
BMI (kg/m^2)	31.1 (23.9–38.6)
HbA$_{1c}$ (mmol/mol)	61.2 ± 9.0‡
Platelet counts (×10^9/L)	227 ± 43
Cholesterol (mmol/L)	
Total	4.14 ± 0.8
LDL	2.54 ± 0.7
HDL	1.07 ± 0.23
Triglycerides (mmol/L)	1.16 ± 0.41
Medications	
Metformin	14 (78)
Insulin	15 (83)
Statin	11 (61)
ACE inhibitor or ARB	10 (55)

Categorical data are shown as number (%) and continuous data as median (range), mean (range) for BMI, or mean ± SD. ARB, angiotensin receptor blocker. *Microvascular complications are retinopathy, neuropathy, or nephropathy. †Defined as drug treatment for hypertension or hyperlipidemia. ‡International Federation of Clinical Chemistry and Laboratory Medicine values, corresponding to 7.75 ± 3.0 with the National Glycohemoglobin Standardization Program/Diabetes Control and Complications Trial method.
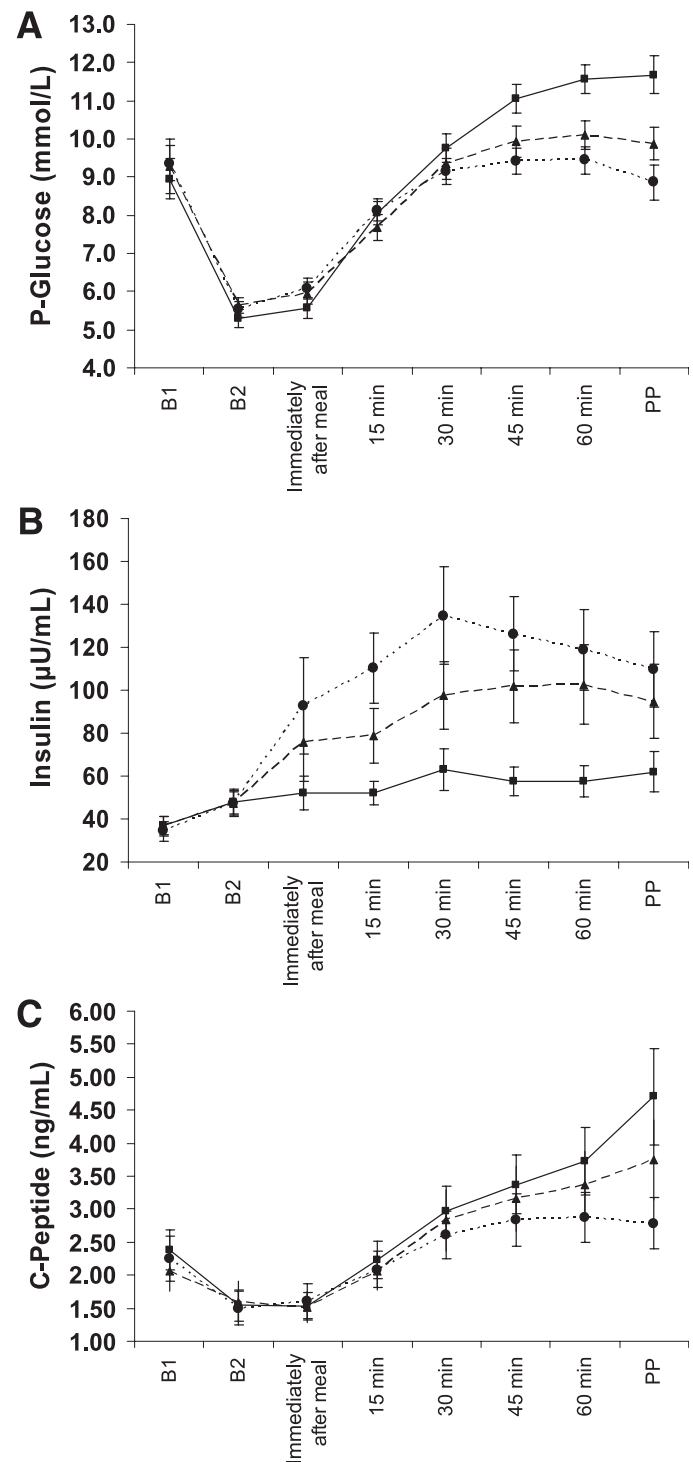
TABLE 2
Glucose, insulin, and C-peptide levels

Variable	Baseline (B1)	Premeal (B2)	After the meal (PP)	P
Glucose (mmol/L)				
Placebo	8.95 ± 2.26	5.29 ± 1.03*	11.68 ± 2.12	
Insulin (0.1 units/kg)	9.28 ± 3.04	5.63 ± 0.84*	9.88 ± 1.79	0.002 vs. placebo
Insulin (0.2 units/kg)	9.37 ± 1.92	5.59 ± 0.89*	8.86 ± 1.95	0.0007 vs. placebo
Insulin (μU/mL)				
Placebo	36.9 ± 17.8	47.9 ± 24.8*	61.9 ± 39.7	
Insulin (0.1 units/kg)	36.8 ± 18.9	47.3 ± 25.0*	94.8 ± 72.4	
Insulin (0.2 units/kg)	34.3 ± 18.9	47.5 ± 22.2*	109.7 ± 74.2	0.04 vs. 0.1 units/kg
C-peptide (ng/mL)				
Placebo	2.38 ± 1.29	1.54 ± 0.99*	4.71 ± 3.09	
Insulin (0.1 units/kg)	2.07 ± 1.32	1.59 ± 1.30*	3.75 ± 2.59	
Insulin (0.2 units/kg)	2.24 ± 1.44	1.50 ± 1.07*	2.79 ± 1.67	<0.001 vs. placebo

Data are presented as mean values ± SD. *$P < 0.05$ compared with B1 (before glucose standardization).

Platelet activation after insulin infusion. Six patients did not require insulin infusion on 11 different occasions (B1 glucose was below 7 mmol/L); these occasions were not included in this analysis. Significant platelet activation occurred after intravenous insulin infusion on 43 occasions. P-selectin–positive platelets were mildly but significantly enhanced by 10% in unstimulated blood (from 2.3 ± 0.6 to 2.6 ± 0.7; $P = 0.005$) and by 7.5% in ADP-stimulated samples (from 56.3 ± 8.0 to 60.6 ± 8.5; $P < 0.001$), whereas marked enhancement was seen with U46619 (67%, from 19.1 ± 13.7 to 31.7 ± 22.1; $P < 0.001$).

Platelet activation after the meal. Platelet activation was enhanced 90 min after the meal (PP), most markedly in response to U46619 stimulation (Fig. 2). Modest but significant meal effects on platelet P-selectin expression were also found in unstimulated and ADP-stimulated samples ($P = 0.006$ and $P < 0.001$, respectively, in the overall ANOVAs). Thus, ADP increased P-selectin expression from 2.3 ± 0.7% to 56.2 ± 8.0% at baseline and from 2.5 ± 0.6% to 62.4 ± 8.1% after the meal on the placebo occasion. GPVI stimulation increased P-selectin expression to ~30% and fibrinogen binding to ~20% on all occasions (i.e., without effects of meal intake or insulin treatment, results not shown).

The following net meal effects on platelet activation (PP vs. B2) could be seen after placebo injection: U46619–stimulated P-selectin expression increased by 23% ($P = 0.02$, Fig. 2A) and ADP-induced P-selectin expression increased by 5% ($P = 0.005$, data not shown). Other measures, such as unstimulated platelet P-selectin expression and fibrinogen binding, did not increase after placebo injection (Fig. 2).

Postprandial platelet activation was much more consistent and pronounced after premeal insulin compared with placebo, with no significant difference between 0.1 and 0.2 units/kg insulin. There was no significant interaction term for treatment effects on platelet P-selectin expression in unstimulated or ADP-stimulated samples, but U46619-stimulated samples showed larger responses after premeal insulin ($P = 0.013$ for interaction term in the ANOVA; Fig. 2A). Postprandial P-selectin expression increased by 10% ($P = 0.01$) and 15% ($P = 0.02$) in unstimulated samples after premeal injections of 0.1 and 0.2 units/kg insulin, respectively. Increases were 57% and 54% in samples stimulated with U46619 ($P < 0.001$ for both; Fig. 2A) and 5% ($P = 0.006$) and 9% ($P < 0.001$) with ADP (data not shown).

Meal effects on platelet fibrinogen-binding were more complex: unchanged in unstimulated samples, slightly but significantly decreased with ADP-stimulation, and increased by 46–49%, on average, in U-46619-stimulated samples after premeal insulin ($P = 0.007$ for meal effects in the ANOVA; $P = 0.045$ for treatment effect; Fig. 2B).

The postprandial enhancement of U46619-induced platelet P-selectin expression was inversely correlated with changes in blood glucose (Fig. 2C) and positively correlated with changes in plasma insulin from B2 to PP (Fig. 2D). Similarly, meal-induced changes in platelet fibrinogen binding tended to correlate inversely with plasma glucose ($P = 0.06$) and positively with plasma insulin ($P = 0.04$; data not shown).

DISCUSSION

This study was designed to evaluate whether platelet activation after a carbohydrate-rich meal is related to the PPH. However, attenuation of PPH by premeal insulin injections did not result in reduced platelet activation. On the contrary, platelet activation evoked by thromboxane receptor stimulation (U46619) was markedly enhanced after premeal insulin, and a positive correlation was observed between postprandial platelet activation and insulin levels. In support of direct or indirect platelet activating effects of insulin in vivo, we found that insulin infusion to standardize premeal glucose levels was also associated with increased platelet reactivity. These results suggest that postprandial hyperinsulinemia, rather than PPH, causes platelet hyperactivity mediated via pathways stimulated by thromboxane and ADP but not collagen after food intake in patients with T2DM.

Several studies, including work from our laboratory, have shown that high glucose can increase platelet reactivity via an elevation of osmolality (11,12). Our findings of postprandial platelet hyperreactivity in T2DM patients but not in healthy control subjects led us to propose that PPH causes platelet dysfunction (5). In line with this notion, acarbose treatment, which attenuates PPH, reduced the urinary thromboxane metabolite excretion in T2DM (6). Our present finding challenges the concept that PPH enhances platelet activation (6) and indicates that other mechanisms may underlie postprandial platelet hyperactivity in T2DM patients.

Platelets express insulin and IGF-1 receptors, which may be activated by insulin (13–15), but the literature on insulin effects on platelet activation is inconsistent. We have found mild platelet-activating effects of insulin at physiologic concentrations (10–100 μU/mL [60–600 pmol/L]) in vitro (9,16). This is in line with the present in vivo results. However,
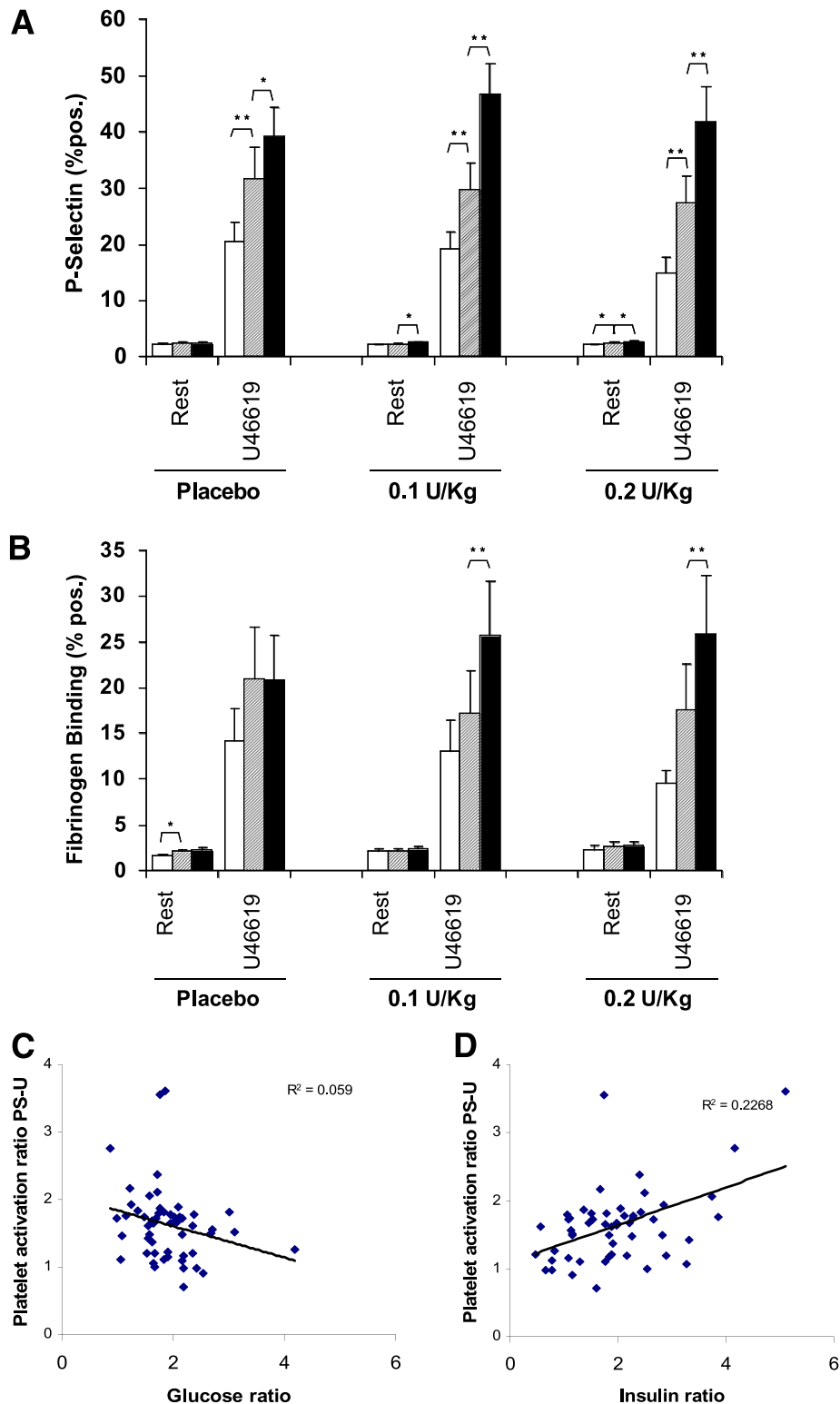
FIG. 2. Platelet P-selectin expression (*A*) and fibrinogen binding (*B*) expressed as the percentage of positive cells without (Rest) and with thromboxane A_2 receptor stimulation by U46619 (0.3 μmol/L) on the three experimental occasions (i.e., with premeal injections of placebo or 0.1 or 0.2 units/kg insulin). Measurements were performed before (B1; □) and after (B2; ▨) glucose standardization, and 90 min after the meal (PP; ■). Mean values and SEM. *$P < 0.05$, **$P < 0.01$. The postprandial platelet activation responses to U46619 were significantly enhanced by meal insulin treatment in the ANOVA. Correlations are shown between postprandial glucose (*C*) and insulin responses (*D*) and platelet P-selectin responses to U46619 (PS-U) stimulation. The postprandial responses were calculated as PP-to-B2 ratios and are shown for all 18 patients on all occasions. The postprandial platelet activation correlated inversely with the postprandial glucose response and positively with the increase in plasma insulin 90 min after the meal. (A high-quality color representation of this figure is available in the online issue.)

several laboratories have found platelet-inhibiting effects of higher insulin concentrations (≥ 1 nmol/L) (14,17,19), and platelet-activating effects of very high insulin concentrations (100 nmol/L) (15,17,19). Others found no direct effects of insulin on platelets in vitro but found inhibitory effects during coincubation with endothelial cells (20). In healthy individuals, insulin appears to suppress platelet activation through inhibition of the P2Y12 pathway (18). However, patients with T2DM and even obese insulin-resistant subjects seem to have "platelet insulin resistance" and lose their responsiveness to insulin (18,21,22). In insulin-treated obese T2DM patients, there are increases in collagen- and ADP-induced platelet adhesion and aggregation, and increased phosphatidylserine exposure (18), which is consistent with our observations.

In the second Diabetes Mellitus Insulin Glucose Infusion in Acute Myocardial Infarction (DIGAMI 2) study, insulin treatment of T2DM patients after a myocardial infarction was associated with an increased risk of myocardial infarction or stroke compared with sulfonylurea treatment, whereas metformin treatment, which increases insulin sensitivity, was associated with a decreased risk compared with sulfonylurea treatment (23). Our findings of platelet activation by insulin in vivo may be relevant in this context. Also of interest are findings of higher platelet activity among T2DM patients receiving dual-antiplatelet treatment who were also treated with insulin compared with those who were not (24). The importance of insulin resistance for these findings in T2DM patients and the mechanisms involved in the presently observed meal effects, such as increased oxidative stress (2), deserve further investigation.

In conclusion, our results suggest that postprandial insulin rather than glucose levels may account for meal-induced platelet hyperactivity in patients with T2DM.

REFERENCES

1. Aryangat AV, Gerich JE. Type 2 diabetes: postprandial hyperglycemia and increased cardiovascular risk. Vasc Health Risk Manag 2010;6:6145–155
2. Monnier L, Mas E, Ginet C, et al. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. JAMA 2006;295:1681–1687
3. Ceriello A, Cavarape A, Martinelli L, et al. The post-prandial state in Type 2 diabetes and endothelial dysfunction: effects of insulin aspart. Diabet Med 2004;21:171–175
4. Yngen M, Östenson CG, Hjemdahl P, Wallén NH. Meal-induced platelet activation in Type 2 diabetes mellitus: effects of treatment with repaglinide and glibenclamide. Diabet Med 2006;23:134–140
5. Razmara M, Hjemdahl P, Yngen M, Ostenson CG, Wallén NH, Li N. Food intake enhances thromboxane receptor-mediated platelet activation in type 2 diabetic patients but not in healthy subjects. Diabetes Care 2007;30:138–140
6. Santilli F, Formoso G, Sbraccia P, et al. Postprandial hyperglycemia is a determinant of platelet activation in early type 2 diabetes mellitus. J Thromb Haemost 2010;8:828–837
7. Woerle HJ, Neumann C, Zschau S, et al. Impact of fasting and postprandial glycemia on overall glycemic control in type 2 diabetes Importance of postprandial glycemia to achieve target HbA1c levels. Diabetes Res Clin Pract 2007;77:280–285
8. Li N, Wallén NH, Hjemdahl P. Evidence for prothrombotic effects of exercise and limited protection by aspirin. Circulation 1999;100:1374–1379
9. Hu H, Li N, Ekberg K, Johansson B-L, Hjemdahl P. Insulin, but not proinsulin C-peptide, enhances platelet fibrinogen binding in vitro in Type 1 diabetes mellitus patients and healthy subjects. Thromb Res 2002;106:91–95
10. Farndale RW, Lisman T, Bihan D, et al. Cell-collagen interactions: the use of peptide Toolkits to investigate collagen-receptor interactions. Biochem Soc Trans 2008;36:241–250
11. Massucco P, Mattiello L, Russo I, et al. High glucose rapidly activates the nitric oxide/cyclic nucleotide pathway in human platelets via an osmotic mechanism. Thromb Haemost 2005;93:517–526
12. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N. High glucose levels enhance platelet activation: involvement of multiple mechanisms. Br J Haematol 2006;133:315–322
13. Hajek AS, Joist JH. Platelet insulin receptor. Methods Enzymol 1992;215:398–403
14. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004;279:3254–3264
15. Hunter RW, Hers I. Insulin/IGF-1 hybrid receptor expression on human platelets: consequences for the effect of insulin on platelet function. J Thromb Haemost 2009;7:2123–2130
16. Yngen M, Li N, Hjemdahl P, Wallén NH. Insulin enhances platelet activation in vitro. Thromb Res 2001;104:85–91
17. Anfossi G, Massucco P, Mattiello L, et al. Insulin exerts opposite effects on platelet function at physiological and supraphysiological concentrations. Thromb Res 1996;82:57–68
18. Ferreira IA, Mocking AIM, Feijge MA, et al. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arterioscler Thromb Vasc Biol 2006;26:417–422
19. Murer EH, Gyda MA, Martinez NJ. Insulin increases the aggregation response of human platelets to ADP. Thromb Res 1994;73:69–74
20. Rauchfuss S, Geiger J, Walter U, Renne T, Gambaryan S. Insulin inhibition of platelet-endothelial interaction is mediated by insulin effects on endothelial cells without direct effects on platelets. J Thromb Haemost 2008;6:856–864
21. Westerbacka J, Yki-Järvinen H, Turpeinen A, et al. Inhibition of platelet-collagen interaction: an in vivo action of insulin abolished by insulin resistance in obesity. Arterioscler Thromb Vasc Biol 2002;22:167–172
22. Gerrits AJ, Koekman CA, van Haeften TW, Akkerman JW. Platelet tissue factor synthesis in type 2 diabetic patients is resistant to inhibition by insulin. Diabetes 2010;59:1487–1495
23. Mellbin LG, Malmberg K, Norhammar A, Wedel H, Rydén L; DIGAMI 2 Investigators. The impact of glucose lowering treatment on long-term prognosis in patients with type 2 diabetes and myocardial infarction: a report from the DIGAMI 2 trial. Eur Heart J 2008;29:166–176
24. Angiolillo DJ, Bernardo E, Ramírez C, et al. Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. J Am Coll Cardiol 2006;48:298–304