Racial Differences in Resistance to P2Y₁₂ Receptor Antagonists in Type 2 Diabetic Subjects[§]

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ABSTRACT

Although resistance to the P2Y₁₂ antagonist clopidogrel is linked to altered drug metabolism, some studies suggest that these pharmacokinetic abnormalities only partially account for drug resistance. To circumvent pharmacokinetic complications and target P2Y₁₂ receptor function we applied the direct P2Y₁₂ antagonist 2-methylthio-AMP (2-methylthioadenosine 5'-monophosphate triethylammonium salt) to purified platelets *ex vivo*. Platelets were purified from healthy and type 2 diabetes mellitus (T2DM) patients and stimulated with thrombin or the selective protease-activated receptor agonists, protease-activated receptor 1-activating peptide (PAR1-AP), or PAR4-AP. Platelet activation as measured by $\alpha_{IIb}\beta_3$ activation, and P-selectin expression was monitored in 141 subjects. Our results demonstrate that, compared with healthy subjects, platelets from diabetic patients are resistant to inhibition by 2-methylthio-AMP, demonstrating P2Y₁₂ pharmacodynamic defects among diabetic patients.

Inhibition of thrombin-mediated $\alpha_{IIb}\beta_3$ activation by 2-methylthio-AMP was lower in diabetic platelets versus healthy platelets. Subgroup analysis revealed a racial difference in the resistance to 2-methylthio-AMP. We found no resistance in platelets from diabetic African Americans; they were inhibited by 2-methylthio-AMP equally as well as platelets from healthy African Americans. In contrast, platelets from Caucasian patients with diabetes were resistant to P2Y₁₂ antagonism compared with healthy Caucasians. Multivariable analysis demonstrated that other variables, such as obesity, age, or gender, could not account for the differential resistance to 2-methylthio-AMP among races. These results suggest that in addition to altered drug metabolism, P2Y₁₂ receptor function itself is altered in the Caucasian diabetic population. The racial difference in platelet function in T2DM is a novel finding, which may lead to differences in treatment as well as new targets for antiplatelet therapy.

Introduction

It is well established that type 2 diabetes mellitus (T2DM) patients display heightened platelet activation and a higher rate of resistance to P2Y₁₂ antagonists (e.g., clopidogrel) (Angiolillo et al., 2005, 2006, 2007; Geisler et al., 2007; Mangiacapra et al., 2010). Dual antiplatelet therapy consisting of aspirin and clopidogrel (the most commonly used P2Y₁₂ receptor antagonist) has become the standard of care in at-risk populations, including T2DM patients, for the treatment of acute coronary

syndrome and following coronary stent implantation. Clopidogrel is a prodrug, and evidence suggests that loss of function in cytochrome P450 results in inadequate transformation to the active metabolite and resultant high residual platelet reactivity (Gladding et al., 2008; Ellis et al., 2009). But cytochrome P450 loss-of-function polymorphisms can only account for a portion of clopidogrel resistance (Shuldiner et al., 2009; Hochholzer et al., 2010). In this study, we specifically address the pharmacodynamic aspects of resistance to P2Y₁₂ antagonists in T2DM. To do so, we studied the effect of a direct P2Y₁₂ antagonist, 2-methylthio-AMP (2-methylthioadenosine 5'-monophosphate triethylammonium salt) (Cusack and Hourani, 1982; Srinivasan et al., 2009; Xiang et al., 2012), in platelet activation.

In vivo, thrombin is a primary activator of platelets, leading to secretion of secondary agonists, including ADP and thromboxane. Thus, we examined resistance to P2Y₁₂ antagonism of thrombin activation of platelets. Most studies examining P2Y₁₂ resistance have used ADP as an agonist (Angiolillo et al., 2005, 2006, 2007; Geisler et al., 2007; Mangiacapra et al., 2010). Examination of responses to the primary activator of platelets, thrombin, is important, as it provides

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ABBREVIATIONS: 2-methylthio-AMP, 2-methylthioadenosine 5'-monophosphate triethylammonium salt; AP, activating peptide; BMI, body mass index; CD62P, P-selectin; PAR, protease-activated receptor; T2DM, type 2 diabetes mellitus.

a view of the role of the P2Y₁₂ receptor in the integrated signaling network of platelets.

Additionally, most studies of P2Y₁₂ resistance were performed in largely male Caucasian populations. We designed our study to include significant numbers of African Americans. Our results indicate that platelet function in Caucasians and African Americans differ; platelets derived from Caucasian T2DM subjects are resistant to inhibitors of P2Y₁₂ receptors, while platelets from African American T2DM patients are not resistant to the effects of P2Y₁₂ receptor inhibitors.

Materials and Methods

Human α -thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). Protease-activated receptor 1-activating peptide (PAR1-AP; SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). FITC-PAC1 (fluorescein isothiocyanate antibody to activated $\alpha_{IIb}\beta_3$) and PE (phycoerythrin)-CD62P (P-selectin) were purchased from BD Pharmingen (San Jose, CA). 2-Methylthio-AMP was purchased from Sigma-Aldrich (St. Louis, MO). Fura-2-AM and probenecid were purchased from Invitrogen (Carlsbad, CA).

Subjects. This study was approved by the University Institutional Review Board. Written informed consent (approved by the Institutional Review Board) was obtained from all individuals prior to blood donation. Inclusion criteria included patients over 18 years of age (male or female) with T2DM or healthy subjects. Exclusion criteria included caffeine or ethanol use 12 hours prior to blood draw, use of P2Y₁₂ antagonists within 10 days prior to blood draw, active use of hormone replacement therapy or corticosteroids, and history of coronary artery disease. Race was self-reported. After blood was drawn and washed platelets were prepared, platelet function was performed as detailed below.

Platelet Preparation. For all studies, washed platelets were resuspended in Tyrode's buffer as previously described (Holinstat et al., 2006, 2007, 2012, 2013).

Surface Expression of P-Selectin and $\alpha_{IIb}\beta_3$ Activation. P-selectin expression (PE-CD62P binding) and $\alpha_{IIb}\beta_3$ activation (FITC-PAC1 binding) were assessed using flow cytometry as previously described (Shattil et al., 1987). Washed platelets were preincubated with CD62P and PAC1 before stimulation with low and high concentrations of thrombin (2 and 10 nM), PAR1-AP (2.5 and 20 μ M), or PAR4-AP (100 and 200 μ M). Prior to stimulation, samples were incubated for 5 minutes with 50 μ M 2-methylthio-AMP or were untreated. Dose-response curves (Fig. 1A) were constructed using washed platelets isolated from normal subjects.

Measurement of Intracellular Ca²⁺ Mobilization. Ca²⁺ mobilization was measured in washed platelets (Santoro et al., 1994; Voss et al., 2007), at a concentration of 2×10^5 platelets/ml. Platelet-rich plasma was incubated with Fura-2-AM (4 μ M) for 60 minutes at 37°C. Subsequently, platelets were pelleted at 700g for 10 minutes and resuspended in Tyrode's containing 2.5 mM probenecid. Prior to stimulation, samples were incubated for 5 minutes with 50 μ M 2-methylthio-AMP or were untreated. Ca²⁺ (2.5 mM final concentration) was added immediately prior to platelet stimulation. Fluorescence measurements were gathered at excitation/emission of 340/510 nm in a Varian fluorometer (Palo Alto, CA) for 10 minutes. The data were analyzed as the increase in fluorescence with agonist relative to an unstimulated control. Data are expressed as maximal Ca²⁺ mobilization and area under the curve.

Statistics. Demographics and medical history data were summarized in terms of median and interquartile range for continuous variables, and frequency and percentage for categorical data. Group comparisons were performed with the use of Wilcoxon rank sum test for continuous variables and Pearson χ^2 test for categorical variables. Multivariable linear regression models, adjusted by age, gender, body mass index (BMI), and medications, were fit to assess the association of diabetes and outcomes of PAC1 or P-selectin. The nonlinear association

Fig. 1. (A) Dose response of 2-methylthio-AMP inhibition of thrombin-mediated P-selectin expression and $\alpha_{IIb}\beta_3$ activation in human platelets. Washed human platelets were stimulated with 10 nM thrombin with increasing concentrations of 2-methylthio-AMP as shown. P-selectin and $\alpha_{IIb}\beta_3$ activation were measured as described in *Materials and Methods*. Data are expressed as the percent of maximum response relative to vehicle-treated control ($n = 3$). (B and C). 2-Methylthio-AMP (50 μ M) does not affect thrombin, PAR1-AP, PAR4-AP, or ADP-mediated intracellular Ca²⁺ mobilization in human platelets. Human platelets were incubated with 50 μ M 2-methylthio-AMP or vehicle for 5 minutes, and agonist was added as indicated (PAR1-AP, 20 μ M; PAR4-AP, 200 μ M; and ADP, 20 μ M). Ca²⁺ mobilization was measured as indicated in *Materials and Methods*. In B, the data are expressed as peak Ca²⁺ response in fold increase in fluorescence relative to baseline. In C, the data are expressed as the area under the curve (AUC). In B and C, $n = 5$.

between a continuous covariate and an outcome variable was characterized by restricted cubic spline. All analyses were performed using statistical software R version 2.13.2 (2011-09-30).

Display of Data. For percent inhibition graphs (bottom panels): * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values are expressed as mean and 95% confidence interval in fold stimulation graphs and mean $\pm$ S.E.M. in percent inhibition graphs.

Results

Table 1 shows the demographics of the 141 recruited subjects stratified according to disease state. Table 2 shows the demographics stratified according to disease state and

TABLE 1

Demographics of healthy and T2DM subjects recruited

Test statistic was performed using the Wilcoxon rank sum test for continuous variables and Pearson χ^2 test for categorical variables. In some cases blood pressure readings were taken from the electronic medical record within 6 months of blood draw.

Clinical Characteristics (<i>n</i> = 141)	Healthy (<i>n</i> = 75)	T2DM (<i>n</i> = 66)	Test Statistic
Age (yrs) ^a	29 44 55	46 54 60	<0.001
Female, <i>n</i> (%)	39 (52.0)	30 (45.5)	0.438
Caucasian, <i>n</i> (%)	49 (65.3)	31 (47.0)	0.028
Hispanic, <i>n</i> (%)	3 (4.0)	3 (4.5)	0.873
BMI ^a	22.7 25.8 30.5	28.0 32.5 37.5	<0.001
SBP (mm Hg) ^a	113.0 120.0 132.0 ^b	120.0 126.0 137.5 ^c	0.092
DBP (mm Hg) ^a	70.0 78.5 83.0 ^b	70.0 78.0 86.0 ^c	0.860
HgA1C (%) ^a	ND	6.40 6.90 8.25	NA
Smokers, <i>n</i> (%)	2 (2.7)	4 (6.1)	0.319
Medications, <i>n</i> (%)			
Aspirin	4 (5.3)	25 (37.9)	<0.001
β -Blocker	4 (5.3)	23 (34.8)	<0.001
ACE inhibitor	4 (5.3)	24 (36.4)	<0.001
ARB	3 (4.0)	7 (10.6)	0.127
Calcium channel blocker	1 (1.3)	10 (15.2)	0.002
Statin	8 (10.7)	35 (53.0)	<0.001
Diuretic	6 (8.0)	21 (31.8)	<0.001
Nitrates	0	5 (7.6)	0.015
Metformin	1 (1.3) ^d	42 (63.6)	<0.001
Insulin	0	20 (30.3)	<0.001
Sulfonylurea	0	13 (19.7)	<0.001
Gliptin	0	4 (6.1)	0.031
Glitazone	0	4 (6.1)	0.031
Incretin mimetic	0	5 (7.6)	0.015
Warfarin	0	5 (7.6)	0.015
NSAID	3 (4.0)	7 (10.6)	0.127
Acetaminophen	2 (2.7)	8 (12.1)	0.029

ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; ASA, aspirin; Cauc, Caucasian; DBP, diastolic blood pressure; NA, not applicable; ND, no data; NSAID, nonsteroidal anti-inflammatory drug; SBP, systolic blood pressure.

^aLower quartile, median quartile, and upper quartile, respectively.

^b*n* of 26.

^c*n* of 31.

^dThe one subject in the healthy group on metformin is taking this for treatment of polycystic ovary syndrome.

race. Washed platelets were stimulated with thrombin, PAR1-AP, or PAR4-AP in the absence or presence of the direct P2Y₁₂ antagonist 2-methylthio-AMP. Agonist concentrations were carefully chosen so that partial and full platelet activation was obtained (Holinstat et al., 2006, 2007). We chose a 50- μ M concentration of 2-methylthio-AMP, as this provided maximal inhibition of thrombin-mediated P-selectin expression and $\alpha_{IIb}\beta_3$ activation in human platelets (Fig. 1A).

The target of 2-methylthio-AMP is P2Y₁₂, a Gi-coupled receptor. However, there is a risk that 2-methylthio-AMP would have off-target effects on P2Y₁. As it is a Gq-coupled receptor, P2Y₁ inhibition during PAR stimulation could affect intracellular Ca²⁺ mobilization that contributes to both secretion (P-selectin expression) and $\alpha_{IIb}\beta_3$ activation. To ensure that an off-target effect of 2-methylthio-AMP on P2Y₁₂ is not contributing to the inhibition of PAR-mediated responses we measured the effect of 2-methylthio-AMP on intracellular Ca²⁺ mobilization in human platelets. Addition of 50 μ M 2-methylthio-AMP did not significantly inhibit thrombin, PAR1-AP, PAR4-AP, or ADP-mediated platelet Ca²⁺ mobilization as demonstrated in Fig. 1, B and C. These data indicate that any observed inhibition of P-selectin expression and $\alpha_{IIb}\beta_3$ activation by 2-methylthio-AMP cannot be accounted for by altered Ca²⁺ mobilization and therefore is probably not attributable to an off-target effect on P2Y₁. Similar results were reported by Xiang et al. (2012), who demonstrated that 2-methylthio-AMP failed to inhibit Ca²⁺ mobilization in P2Y₁₂-deficient mouse platelets and did not

raise cAMP or induce vasodilator-stimulated phosphoprotein phosphorylation in wild-type platelets, demonstrating that 2-methylthio-AMP inhibits platelet function through a P2Y₁₂-dependent mechanism.

Effects of Direct P2Y₁₂ Inhibition on $\alpha_{IIb}\beta_3$ Activation in Thrombin-Stimulated Platelets. $\alpha_{IIb}\beta_3$ activation (PAC1 binding) stimulated by thrombin, PAR1-AP, or PAR4-AP, was measured in the presence or absence of 2-methylthio-AMP. The data are expressed as a fold increase above basal in Fig. 2; the fold increases of PAC1 binding were similar regardless of the presence or absence of T2DM (Fig. 2, A, C, and E). In healthy subjects, fold increase in PAC1 binding in response to low thrombin (2 nM) was reduced from 7.1 ± 0.6 to 3.2 ± 0.3 (49.3 $\pm$ 2.8% inhibition; Fig. 2B) by 2-methylthio-AMP. In T2DM subjects, 2-methylthio-AMP was less effective at inhibiting $\alpha_{IIb}\beta_3$ activation by thrombin. In these subjects, fold increase was reduced from 8.5 ± 0.6 to 5.3 ± 0.5 (27.3 $\pm$ 6.9% inhibition; Fig. 2B) in the presence of 2-methylthio-AMP. The discrepancy in the level of inhibition obtained with 2-methylthio-AMP between platelets from healthy subjects and those from T2DM patients was more evident when platelets were stimulated with high thrombin (10 nM): 13.0 ± 0.7 to 7.3 ± 0.4 fold increase (40.8% inhibition; Fig. 2B) in healthy subjects and 10.8 ± 0.7 to 8.3 ± 0.6 fold increase (20.3% inhibition; Fig. 2B) in T2DM subjects. This significant difference in 2-methylthio-AMP-mediated inhibition of $\alpha_{IIb}\beta_3$ activation was also observed with high-dose PAR1-AP, but not with low-dose PAR1-AP

TABLE 2

Demographics of healthy subjects compared with T2DM patients stratified by Race

Test statistic was performed using the Wilcoxon rank sum test for continuous variables and Pearson χ^2 test for categorical variables. In some cases blood pressure readings were taken from the Electronic Medical Record and were obtained within 6 months of blood draw.

Clinical Characteristics	Healthy Cauc (n = 49)	T2DM Cauc (n = 31)	Test Statistic	Healthy AA (n = 26)	T2DM AA (n = 35)	Test Statistic
Age (yrs) ^a	28 46 58	52 55 62	0.007	30 42.5 50	45 51 59	0.003
Female, n (%)	29 (59.2)	14 (45.2)	0.22	10 (38.5)	16 (45.7)	0.571
Hispanic, n (%)	3 (6.1)	1 (3.2)	0.562	0	2 (5.7)	0.215
BMI ^a	22.5 25.1 29.3	28.8 33.2 40.8	<0.001	24.1 25.8 31.1	28.1 32.2 36.7	0.002
SBP (mm Hg) ^a	112.5 120.0 125.5 ^b	120 123.0 135 ^c	0.125	122 132 136.5 ^d	125 134 139.5 ^e	0.524
DBP (mm Hg) ^a	70 79 84 ^b	70 79 86 ^c	0.971	69.5 72 79 ^d	69 75 85.5 ^e	0.724
HgA1C (%) ^a	ND	6.3 6.7 7.3	NA	ND	6.8 7.4 10.6	NA
Smokers, n (%)	1 (2.0)	2 (6.5)	0.312	1 (3.8)	2 (5.7)	0.739
Medications, n (%)						
Aspirin	3 (6.1)	12 (38.7)	<0.001	1 (3.8)	13 (37.1)	0.002
β -Blocker	2 (4.1)	11 (35.5)	<0.001	2 (7.7)	12 (34.3)	0.015
ACE Inhibitor	3 (6.1)	11 (35.5)	<0.001	1 (3.8)	13 (37.1)	0.002
ARB	2 (4.1)	3 (9.7)	0.314	1 (3.8)	4 (11.4)	0.286
Calcium channel blocker	0	4 (12.9)	0.01	1 (3.8)	6 (17.1)	0.107
Diuretic	3 (6.1)	7 (22.6)	0.03	3 (11.5)	14 (40.0)	0.014
Nitrates	0	2 (6.5)	0.072	0	3 (8.6)	0.126
Metformin	0	23 (74.2)	<0.001	1 (3.8)	19 (54.3)	<0.001
Insulin	0	7 (22.6)	<0.001	0	13 (37.1)	<0.001
Sulfonylurea	0	5 (16.1)	0.004	0	8 (22.9)	0.009
Gliptin	0	0	NA	0	4 (11.4)	0.075
Glitazone	0	3 (9.7)	0.026	0	1 (2.9)	0.385
Incretin mimetic	0	4 (12.9)	0.01	0	1 (2.9)	0.385
Warfarin	0	2 (6.5)	0.072	0	3 (8.6)	0.126
NSAID	2 (4.1)	4 (12.9)	0.144	1 (3.8)	3 (8.6)	0.461
Acetaminophen	1 (2.0)	3 (9.7)	0.127	1 (3.8)	5 (14.3)	0.176

AA, African American; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; Cauc, Caucasian; DBP, diastolic blood pressure; NA, not applicable; ND, no data; NSAID, nonsteroidal anti-inflammatory drug; SBP, systolic blood pressure.

^aLower quartile, median quartile, and upper quartile, respectively.^bn of 21.^cn of 23.^dn of 5.^en of 8.

(Fig. 2D), and with both high and low PAR4-AP-stimulated platelets (Fig. 2F).

The large percentage of African-American subjects enrolled permitted subgroup analysis to examine effects of P2Y₁₂-inhibition in African-American T2DM subjects, which has been largely unstudied. While platelets from Caucasian T2DM subjects (compared with Caucasian healthy subjects) were resistant to inhibition by 2-methylthio-AMP, platelets from African American subjects were inhibited similarly in both T2DM and healthy groups (Fig. 3). 2-Methylthio-AMP inhibited low-dose thrombin-stimulated $\alpha_{IIb}\beta_3$ activation in platelets from Caucasian subjects by 54.9%, compared with 9.0% in platelets from T2DM Caucasian subjects (Fig. 3B), a significant resistance to P2Y₁₂ antagonism. In contrast, 2-methylthio-AMP inhibited low-dose thrombin-stimulated $\alpha_{IIb}\beta_3$ activation in platelets from African-American subjects by 38.9%, while platelets from African-American T2DM subjects were inhibited by 43.6% (Fig. 3B). Similar results were obtained when platelets were stimulated with PAR1-AP or PAR4-AP (Fig. 4).

Effects of Direct P2Y₁₂-Inhibition on α -Granule Secretion in Thrombin-Stimulated Platelets. As an additional measure of platelet activation, we also assessed the effect of 2-methylthio-AMP on α -granule secretion in response to PAR stimulation. To evaluate the effects of 2-methylthio-AMP on α -granule secretion, surface expression of P-selectin was assessed by CD62P binding as measured by flow cytometry (Fig. 5). Results paralleled those observed with the $\alpha_{IIb}\beta_3$ activation. Inhibition of 2 nM thrombin-mediated P-selectin

expression by 2-methylthio-AMP was greater in platelets from healthy-versus-T2DM subjects ($41.5 \pm 2.8\%$ inhibition versus $28.4 \pm 5.5\%$, respectively; Fig. 5A). With a maximal dose of thrombin (10 nM), greater inhibition with 2-methylthio-AMP was again observed in platelets from healthy subjects compared with T2DM subjects ($31.8 \pm 2.3\%$ versus $16.8 \pm 3.0\%$; Fig. 5A). Similar results were obtained when platelets were stimulated with PAR1-AP and PAR4-AP (Fig. 5, B and C).

As with $\alpha_{IIb}\beta_3$ activation, PAR-mediated P-selectin surface expression on platelets from Caucasian T2DM subjects was resistant to inhibition by 2-methylthio-AMP compared with Caucasian healthy subjects (Figs. 6 and 7, A and B, respectively). On the other hand, platelets from African-American T2DM subjects respond to 2-methylthio-AMP with percent inhibition values similar to those observed in healthy African-American subjects (Figs. 6 and 7).

Multivariable Analysis. It is possible that factors other than race and diabetic status contribute to the trends observed in this analysis. Demographic and pharmacologic differences among the healthy and T2DM subjects potentially could affect platelet function (Table 2). Therefore, we performed multivariable analysis of the data. Multivariable linear regression models for all parameters examined (including age, race, sex, BMI, aspirin, insulin, β -blockers, and diuretics) indicated that, with respect to $\alpha_{IIb}\beta_3$ activation, none except race could account for the difference in sensitivity to 2-methylthio-AMP observed on platelets isolated from African-American T2DM and Caucasian T2DM subjects. With respect to P-selectin expression, multivariable linear regression models for all parameters

Fig. 2. Inhibition of agonist-mediated $\alpha_{IIb}\beta_3$ activation in platelets by 2-methylthio-AMP. (A) Thrombin: scatter plot depicting fold stimulation of $\alpha_{IIb}\beta_3$ of platelets stimulated by thrombin from all subjects grouped together according to T2DM status. (B) Thrombin: percent inhibition by 2-methylthio-AMP of platelets stimulated with thrombin plotted on the y-axis with mean and S.E.M. shown. Low and high doses were 2 and 10 nM thrombin, respectively. (C) PAR1-AP: scatter plot depicting fold stimulation of $\alpha_{IIb}\beta_3$ activation of platelets stimulated with PAR1-AP. (D) PAR1-AP: percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR1-AP plotted on the y-axis with mean and S.E.M. shown. Low and high doses were 2.5 and 20 μM PAR1-agonist peptide, respectively. (E) PAR4-agonist peptide: scatter plot depicting fold stimulation of $\alpha_{IIb}\beta_3$ activation of platelets stimulated by PAR4-AP. (F) PAR4-AP: percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-AP with mean and S.E.M. shown. Low and high doses were 100 and 200 μM PAR4-AP, respectively. * $P < 0.05$; *** $P < 0.001$. 2MeS, 2-methylthio-AMP.

Fig. 3. Racial differences in inhibition of thrombin-mediated $\alpha\text{IIb}\beta_3$ activation in platelets by 2-methylthio-AMP. (A) Scatter plot depicting fold stimulation of $\alpha\text{IIb}\beta_3$ activation of platelets stimulated with thrombin from subjects stratified on basis of racial background as well as T2DM status. (B) Percent inhibition with 2-methylthio-AMP from subjects stratified on basis of race and T2DM status plotted on the y-axis with mean and S.E.M. shown. Low and high doses were 2 and 10 nM thrombin, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. 2MeS, 2-methylthio-AMP; AA, African American; Cauc, Caucasian.

(including age, race, sex, BMI, aspirin, insulin, β -blockers, and diuretics) indicated that none could account for the difference in sensitivity to 2-methylthio-AMP observed in platelets isolated from African-American T2DM and Caucasian

T2DM subjects with one exception: BMI associated with high-dose thrombin-mediated P-selectin expression ($P = 0.022$), but not with low-dose thrombin or with any dose of PAR1-AP or PAR4-AP.

Fig. 4. Racial differences in inhibition of PAR1-AP-mediated α IIb β 3 activation in platelets by 2-methylthio-AMP. (A) Percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR1-AP from subjects stratified on basis of race and T2DM status plotted on the y-axis with mean and S.E.M. shown. Low and high doses were 2.5 and 20 μ M PAR1-AP, respectively. (B) Percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-AP from subjects stratified on basis of race and T2DM status plotted on the y-axis with mean and S.E.M. shown. Low and high doses were 100 and 200 μ M PAR4-AP, respectively. * P < 0.05; ** P < 0.01; *** P < 0.001. AA, African American, Cauc, Caucasian.

Discussion

The P2Y₁₂ receptor antagonist clopidogrel is widely used for prevention of vascular ischemic events in patients with thrombotic risk by inhibiting P2Y₁₂-mediated activation of platelets. ADP is released from platelet-dense granules upon

stimulation with primary agonists, such as thrombin and collagen (Rao, 1990; Gachet, 2006). Our data confirm that a component of the platelet response to thrombin is provided by ADP secretion and autocrine action on the P2Y₁₂ receptor. P2Y₁₂ inhibition has been shown to decrease thrombin-stimulated platelet aggregation

Fig. 5. Inhibition of agonist-mediated P-selectin activation in platelets by 2-methylthio-AMP. (A) Thrombin: percent inhibition with 2-methylthio-AMP of platelets stimulated with thrombin from subjects stratified on the basis of T2DM status with mean and S.E.M. shown. Low and high doses were 2 and 10 nM thrombin, respectively. (B) PAR1-AP: percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR1-AP from subjects stratified on basis of T2DM status plotted on the y-axis with mean and S.E.M. shown. Low and high doses were 2.5 and 20 μ M PAR1-AP, respectively. (C) PAR4-AP: percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-AP from subjects stratified on basis of T2DM status with mean and S.E.M. shown. Low and high doses were 100 and 200 μ M PAR4-AP, respectively. * P < 0.05; ** P < 0.01; *** P < 0.001.

and activation in normal subjects (Behan et al., 2005). While the effect of diabetes on ADP-induced platelet aggregation and activation is well described (Angiolillo et al., 2005, 2006, 2007; Geisler et al., 2007; Mangiacapra et al., 2010), the effect of diabetes on thrombin-mediated platelet signaling is sparse. Given that ADP is secreted subsequent to PAR activation and the substantial contributions of P2Y₁₂ stimulation to the full thrombin response, there is a need to understand how diabetes affects the coordinated signaling between thrombin and ADP

on the platelet. For example, only one group has reported that P2Y₁₂ antagonism of PAR-1-mediated activation of platelets is attenuated in the presence of diabetes (Angiolillo et al., 2007). Moreover, this group only examined PAR-1-mediated platelet activation (along with ADP, collagen, and epinephrine) at one concentration (25 μ M); thus, they did not investigate if P2Y₁₂ antagonism of thrombin or PAR4-AP-mediated platelet activation is also attenuated in diabetes. Our data confirm this finding and extend the observation to the physiologic activator

Fig. 6. Racial differences in inhibition of thrombin-mediated P-selectin activation in platelets by 2-methylthio-AMP. Percent inhibition with 2-methylthio-AMP of platelets stimulated by thrombin from subjects stratified on basis of T2DM status and race and with mean and S.E.M. shown. Low and high doses were 2 and 10 μ M thrombin, respectively. * P < 0.05; ** P < 0.01; *** P < 0.001. AA, African American, Cauc, Caucasian.

Fig. 7. Racial differences in inhibition of PAR1-AP-mediated P-selectin activation in platelets by 2-methylthio-AMP. (A) Percent inhibition with 2-methylthio-AMP of platelets stimulated by PAR1-AP from subjects stratified on basis of T2DM status and race and with mean and S.E.M. shown. Low and high doses were 2.5 and 20 μ M PAR1-AP, respectively. (B) Percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-AP from subjects stratified on basis of T2DM status and race and with mean and S.E.M. shown. Low and high doses were 100 and 200 μ M PAR4-AP, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. AA, African American, Cauc, Caucasian.

thrombin and PAR4-AP stimulation of platelets (Yee et al., 2005; Angiolillo et al., 2007; Gori et al., 2008). More importantly, we have identified a racial difference in the platelet response to direct P2Y₁₂ antagonists that has never been reported.

The importance of platelet activation in the development of atherothrombosis is reflected by the benefits of aspirin, P2Y₁₂ antagonists, and $\alpha_{IIb}\beta_3$ inhibitors in the treatment of atherothrombosis. However, many patients are resistant to

clopidogrel (Nguyen et al., 2005), and multiple studies have observed that high residual platelet reactivity during clopidogrel treatment leads to increased risk of stent thrombosis and cardiac complications (Matetzky et al., 2004; Gurbel et al., 2005; Cheng et al., 2006; Cuisset et al., 2006, 2007; Hochholzer et al., 2006; Lev et al., 2006). Pharmacokinetic studies focus on metabolism of the prodrug clopidogrel as a mechanism of resistance (Gladding et al., 2008; Ellis et al., 2009); however, these studies did not account for altered platelet P2Y₁₂ receptor number or function (pharmacodynamic effects), increased circulating ADP, and upregulation of components of the P2Y₁₂-receptor signaling cascade. As we observed a similar resistance to P2Y₁₂ antagonism utilizing a direct P2Y₁₂ antagonist in ex vivo platelets, we hypothesize that resistance to clopidogrel in T2DM patients (Matetzky et al., 2004; Angiolillo et al., 2005; Cuisset et al., 2006) is attributable at least in part to pharmacodynamic complications at the level of the receptor or its downstream signaling pathways. Based on the current data we can only speculate about the mechanism. Given that the difference in response was observed between racial groups with T2DM, it seems likely that there is a genetic component that manifests in the context of the pathophysiology associated with T2DM. For example, there may be differences in receptor internalization, desensitization, or resensitization. In this scenario, high circulating ADP levels in diabetic subjects could lead to different densities of P2Y₁₂ receptors on the platelet surface, potentially creating a larger receptor reserve in one group versus another. Recently, a study that measured the active metabolite of clopidogrel and also excluded subjects with CYP2C19 loss-of-function polymorphisms along with CYP3A5, ABC1, and PON1 polymorphisms while rigorously controlling for other demographics that influence platelet reactivity concluded that unidentified factors contribute to high on-treatment platelet reactivity (Frelinger et al., 2013).

One question that arises is whether the effect observed with 2-methylthio-AMP is attributable to the blockage of endogenous ADP and its contribution to thrombin-mediated platelet activation or if the P2Y₁₂ receptor is constitutively active. We did not test the effect of apyrase on each individual in this study. However, previously it has been shown that after removal of ADP and its metabolites by addition of apyrase or adenosine deaminase, PAR1-AP-mediated platelet activation remains unaltered in the presence of P2Y₁₂ antagonists (Iyu et al., 2011), suggesting there is little to no constitutive activity.

Limitations of our study include the necessity of excluding patients who were taking P2Y₁₂ inhibitors. Future studies will specifically address the influence of coronary artery disease and if African-American T2DM subjects display normal inhibition of thrombin-mediated responses to oral P2Y₁₂ inhibitors. One study has demonstrated that 2-methylthio-AMP is structurally distinct from the oral thienopyridine class of P2Y₁₂-antagonists (e.g., clopidogrel) and has a different mechanism of action (Srinivasan et al., 2009), which limits the direct application of our finding to clinical practice. However, more recently it was reported that 2-methylthio-AMP does inhibit the P2Y₁₂ receptor (Xiang et al., 2012). Regardless, the potential unique mechanism of action of the P2Y₁₂ antagonist 2-methylthio-AMP does not detract from the pharmacodynamic conclusion that P2Y₁₂ receptor function is altered in the T2DM population, particularly among Caucasians, and instead spurs the need for further investigation of this phenomenon.

Of note, when measuring platelet aggregation we did not observe any significant differences in P2Y₁₂ antagonism in

responses with respect to T2DM or racial status (Supplemental Fig. 1), which we believe is owing to multiple pathways of feed-forward signaling leading to irreversibility of platelet aggregation. Multiple methods can be used to study platelet response to clopidogrel, in addition to light transmission platelet aggregation. These include flow cytometry analysis of $\alpha_{IIb}\beta_3$ activation and P-selectin surface expression (Gurbel et al., 2007; Bonello et al., 2010). In fact, it has been recognized that flow cytometry is particularly useful to assess pharmacological effects (Gurbel et al., 2007).

We defined race based on self-reporting. A recent investigation has shown that self-designation of race approximates genetic ancestry (Dumitrescu et al., 2010). Therefore, we hypothesize that underlying genetic differences between African Americans and Caucasians are responsible for our observed difference. It is well established that clinical differences in response to the effect of both beta blockers and diuretics exist between different racial groups. In the landmark Veterans Administration Cooperative Study Group on Antihypertensive Agents (1982), it was observed that Caucasians had a greater blood pressure-lowering effect with β -blockers compared with African-American patients, while African Americans had a better response to diuretics compared with Caucasians. These differences in antihypertensive response by race have guided therapy based on race (Messerli and Ventura, 1985; Zing et al., 1991) and have influenced guidelines for treatment (Chobanian et al., 1988). Pharmacogenetic contributions are responsible for some of the racial differences in response to cardiovascular drugs, which has the potential for further personalization of care (Johnson, 2008). Future studies will determine the underlying genetic or metabolic factors between the marked difference in response to P2Y₁₂ inhibition observed between African-American and Caucasian T2DM subjects.

To date no investigations have examined resistance to platelet inhibition by antiplatelet therapeutics in an African-American population. In fact, out of the 8829 samples collected as part of the International Clopidogrel Platelet Consortium, which comprises multiple studies investigating the pharmacogenomics of resistance to antiplatelet agents, only 77 of the subjects are of African-American descent and only 33 of these subjects have T2DM (Alan Shuldiner, personal communication to J.H.C.). Currently two trials are specifically investigating antiplatelet response in African-American populations. A pharmacodynamic study with ticagrelor in African-American patients with stable coronary artery disease (ClinicalTrials.gov Identifier: NCT01523392) has been completed but is not yet published. In addition, the African-American Pharmacogenetics study (ClinicalTrials.gov Identifier: NCT01408121) is a prospective genetic and platelet reactivity cohort study of African Americans and Caucasians undergoing coronary intervention who have received either clopidogrel or prasugrel has been completed but is not yet published.

The observation that platelets from T2DM subjects exhibit resistance to P2Y₁₂ antagonists when stimulated with thrombin, PAR1-AP, or PAR4-AP is an important observation that warrants further study, given the growing population of T2DM patients in the United States. This is especially true considering that, compared with the general public, African Americans are disproportionately affected and have a higher incidence of T2DM (<http://www.cdc.gov/nchs/data/has/has09.pdf>). The differences observed between Caucasian and African-American populations coupled with the direct action of 2-methylthio-AMP

on P2Y₁₂ indicate that there may be differences in platelet P2Y₁₂ receptor function or signaling in these populations. Furthermore, these results urge the necessity for further study of this phenomenon as antagonism of P2Y₁₂ continues to be a common target for the prevention of thrombosis. Our research potentially lays the groundwork for determining the genetic basis of protection from resistance in African-American patients with diabetes as well as for future larger clinical outcome studies in African-Americans undergoing antiplatelet therapy with clopidogrel or other antagonists of P2Y₁₂ receptors.

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Authorship Contributions

Participated in research design: Cleator, Duvernay, Holinstat, Hamm.

Conducted experiments: Duvernay, Holinstat, Colowick, Hudson.

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Wrote or contributed to the writing of the manuscript: Cleator, Duvernay, Hamm.

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