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 cAMP 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 cAMP 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.

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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 P2Y<sub>12</sub> receptor in the integrated
signaling network of platelets.

Additionally, most studies of P2Y<sub>12</sub> 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 P2Y<sub>12</sub> receptors,
while platelets from African American T2DM patients are not
resistant to the effects of P2Y<sub>12</sub> receptor inhibitors.

**Materials and Methods**

Human α-thrombin was purchased from Enzyme Research Labora-
tories (South Bend, IN). Protease-activated receptor 1–activating
peptide (PAR1-AP; SFLRN) and PAR4-AP (AYPGKF) were
purchased from GL Biochem (Shanghai, China). FITC-PAC1 (fluores-
cence isothiocynate antibody to activated α<sub>IIb</sub>β<sub>3</sub>) 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 Institu-
tional 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
P2Y<sub>12</sub> 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

**Surface Expression of P-Selectin and α<sub>IIb</sub>β<sub>3</sub> Activation.** P-selectin
expression (PE-CD62P binding) and α<sub>IIb</sub>β<sub>3</sub> activation (FITC-PAC1 binding)
were assayed 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 μM), or PAR4-AP (100 and 200 μM). Prior to
stimulation, samples were incubated for 5 minutes with 50 μM
2-methylthio-AMP or were untreated. Dose-response curves (Fig. 1A)
were constructed using washed platelets isolated from normal subjects.

**Measurement of Intracellular Ca<sup>2+</sup> Mobilization.** Ca<sup>2+</sup>
mobilization was measured in washed platelets (Santoro et al., 1994; Voss
et al., 2007), at a concentration of 2 x 10<sup>8</sup> platelets/ml. Platelet-rich
plasma was incubated with Fura-2-AM (4 μM) 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 μM
2-methylthio-AMP or were untreated. Ca<sup>2+</sup> (2.5 mM final concentra-
tion) 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 Ca<sup>2+</sup> mobiliza-
tion and area under the curve.

**Statistics.** Demographics and medical history data were summa-
rized 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<sup>2</sup> test for categorical variables.
Multivariable linear regression models, adjusted by age, gender, body
mass index (BMI), and medications, were fit to access the association
diabetes and outcomes of PAC1 or P-selectin. The nonlinear association
between a continuous covariate and an outcome variable was character-
ized by restricted cubic spline. All analyses were performed using

**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 ±
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

**Fig. 1.** (A) Dose response of 2-methylthio-AMP inhibition of thrombin-
mediated P-selectin expression and α<sub>IIb</sub>β<sub>3</sub> 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
α<sub>IIb</sub>β<sub>3</sub> 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 μM) does not
affect thrombin, PAR1-AP, PAR4-AP, or ADP-mediated intracellular
Ca<sup>2+</sup> mobilization in human platelets. Human platelets were incubated
with 50 μM 2-methylthio-AMP or vehicle for 5 minutes, and agonist was
added as indicated (PAR1-AP, 20 μM; PAR4-AP, 200 μM; and ADP, 20 μM).
Ca<sup>2+</sup> mobilization was measured as indicated in Materials and Methods.
In B, the data are expressed as peak Ca<sup>2+</sup> 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.
Effects of Direct P2Y12 Inhibition on αP2Y12 β3 Activation in Thrombin-Stimulated Platelets. αP2Y12 β3 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 ± 2.8% inhibition; Fig. 2B) by 2-methylthio-AMP. In T2DM subjects, 2-methylthio-AMP was less effective at inhibiting P2Y12-dependent mechanism.

TABLE 1
Demographics of healthy and T2DM subjects recruited

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Healthy (n = 75)</th>
<th>T2DM (n = 66)</th>
<th>Test Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>29.44 ± 55.5</td>
<td>46.54 ± 60.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>39 (52.0)</td>
<td>30 (45.5)</td>
<td>0.438</td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>49 (65.3)</td>
<td>31 (47.0)</td>
<td>0.028</td>
</tr>
<tr>
<td>Hispanic, n (%)</td>
<td>3 (4.0)</td>
<td>3 (4.5)</td>
<td>0.573</td>
</tr>
<tr>
<td>BMI</td>
<td>22.7 ± 25.8</td>
<td>30.5 ± 12.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>130.0 ± 120.0</td>
<td>120.0 ± 137.5</td>
<td>0.092</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>70.0 ± 83.0</td>
<td>70.0 ± 86.0</td>
<td>0.860</td>
</tr>
<tr>
<td>HgA1C (%)</td>
<td>ND</td>
<td>6.40 ± 8.25</td>
<td>NA</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>2 (2.7)</td>
<td>4 (6.1)</td>
<td>0.319</td>
</tr>
</tbody>
</table>

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

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 ± 2.8% inhibition; Fig. 2B) by 2-methylthio-AMP. In T2DM subjects, 2-methylthio-AMP was less effective at inhibiting P2Y12-dependent mechanism.

The target of 2-methylthio-AMP is P2Y12, a Gq-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 αP2Y12 β3 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 μM 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 αP2Y12 β3 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.
exhibited low-dose thrombin inhibition in African-American T2DM subjects, which has been largely unstudied. While platelets from Caucasian T2DM 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 αIIbβ3 activation in platelets from Caucasian subjects by 54.9%, compared with healthy-versus-T2DM subjects (31.8% versus 16.8%, 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 ± 2.3% versus 16.8 ± 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 αIIbβ3 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 aspirin, insulin, β-blockers, and diuretics) indicated that, with

### TABLE 2

Demographics of healthy subjects compared with T2DM patients stratified by Race

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Healthy Cauc (n = 49)</th>
<th>T2DM Cauc (n = 31)</th>
<th>Test Statistic</th>
<th>Healthy AA (n = 26)</th>
<th>T2DM AA (n = 35)</th>
<th>Test Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>25 ± 8</td>
<td>25 ± 8</td>
<td>0.007</td>
<td>30 ± 12 ± 5.5</td>
<td>45 ± 10 ± 5.5</td>
<td>0.003</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>34 (65.3)</td>
<td>21 (67.7)</td>
<td>0.22</td>
<td>10 (38.5)</td>
<td>16 (45.7)</td>
<td>0.571</td>
</tr>
<tr>
<td>Hispanic, n (%)</td>
<td>3 (6.1)</td>
<td>1 (3.2)</td>
<td>0.562</td>
<td>2 (5.7)</td>
<td>2 (5.7)</td>
<td>0.215</td>
</tr>
<tr>
<td>BMI</td>
<td>22.5 ± 1.2</td>
<td>28.8 ± 3.2</td>
<td>&lt;0.001</td>
<td>29.4 ± 3.1</td>
<td>31.1 ± 3.2</td>
<td>0.002</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>112.5 ± 120.0 ± 125.5</td>
<td>120 ± 123.0 ± 135</td>
<td>0.125</td>
<td>121 ± 132 ± 136.5</td>
<td>125 ± 134 ± 139.5</td>
<td>0.524</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>79 ± 79</td>
<td>79 ± 79</td>
<td>0.971</td>
<td>69 ± 72 ± 79.4</td>
<td>69 ± 75 ± 85.5</td>
<td>0.724</td>
</tr>
<tr>
<td>HgA1C (%)</td>
<td>6.3 ± 0.7 ± 7.3</td>
<td>NA</td>
<td>NA</td>
<td>6.8 ± 7.4 ± 10.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>1 (2.0)</td>
<td>2 (6.5)</td>
<td>0.312</td>
<td>1 (3.8)</td>
<td>2 (5.7)</td>
<td>0.739</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td>3 (6.1)</td>
<td>12 (38.7)</td>
<td>&lt;0.001</td>
<td>1 (3.8)</td>
<td>13 (37.1)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

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.

*Lower quartile, median quartile, and upper quartile, respectively.

*8

*26

*23

*21

*49

*31

*20

*26

*26

*26

*20

*20

*125 134 139.5

*13 137 37.1

*13 22.9

*4 (11.4)

*0 (4.129)

*4 (12.9)

*2 (7.7)

*12 (34.3)

*0 (3.8)

*6 (16.1)

*3 (11.5)

*4 (11.4)

*3 (12.9)

*1 (3.8)

*13 137 37.1

*10 (38.5)
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 nM 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 nM PAR4-AP, respectively. $^*P < 0.05; ^{***}P < 0.001$. 2MeS, 2-methylthio-AMP.
(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. 3.** Racial differences in inhibition of thrombin-mediated α\(_{IIb}\)β\(_{3}\) activation in platelets by 2-methylthio-AMP. (A) Scatter plot depicting fold stimulation of α\(_{IIb}\)β\(_{3}\) 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.
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. 4. Racial differences in inhibition of PAR1-AP–mediated \( \alpha_{IIb} \beta_{3} \) 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 \( \mu \)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 \( \mu \)M PAR4-AP, respectively. *\( P < 0.05; ** P < 0.01; *** P < 0.001 \). AA, African American, Cauc, Caucasian.
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 μM); 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. 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 μM 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 μM PAR4-AP, respectively. *P < 0.05; **P < 0.01; ***P < 0.001.

**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 μM thrombin, 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 α1bβ3 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 sensitization. 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 ABC1, and PON1 polymorphisms while rigorously controlling for demographic differences in receptor internalization, desensitization, or sensitization. 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 ABC1, and PON1 polymorphisms while rigorously controlling for other demographics that influence platelet reactivity concluded that 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 αIβ3 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/hus/hus09.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

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

References


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Racial differences in resistance to P2Y12 receptor antagonists in Type-2 diabetic subjects

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Supplemental Figure 1. Non-linear 2Me-SAMP inhibition of Thrombin-mediated platelet aggregation. Left Panel is a scatter plot depicting fold stimulation of platelet aggregation on the Y axis in all patient stratified according to T2DM status. Right Panel represents subjects stratified on basis of racial background and presence or absence of T2DM and depicts percent inhibition with 2MeSAMP plotted on the Y-axis with mean and SEM shown.