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

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Abbreviations: ADP-Adenosine diphosphate, BMI-Body Mass Index, T2DM-Type 2 Diabetes Mellitus, AA-African-American, and PAR-Protease Activated Receptor.

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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 to purified platelets, ex vivo. Platelets were purified from healthy and Type-2 Diabetes (T2DM) patients and stimulated with thrombin or the selective protease activated receptor agonists, PAR1-activating peptide (PAR1-AP) or PAR4-AP. Platelet activation as measured by αIIbβ3 activation and P-selectin expression was monitored in 141 subjects. Our results demonstrate that compared to healthy subjects, platelets from diabetic patients are resistant to inhibition by 2-methylthio-AMP, demonstrating P2Y12 pharmacodynamic defects amongst diabetic patients. Inhibition of thrombin-mediated αIIbβ3 activation by 2-methylthio-AMP was lower in diabetic platelets versus healthy platelets. Sub-group 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 diabetics were resistant to P2Y12 antagonism compared to 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 potentially lead to new targets for anti-platelet therapy.
Introduction

It is well established that T2DM patients display heightened platelet activation and a higher rate of resistance to P2Y12-antagonists (e.g., clopidogrel) (Angiolillo et al., 2005; Angiolillo et al., 2006; Angiolillo et al., 2007; Geisler et al., 2007; Mangiacapra et al., 2010). Dual anti-platelet 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, for the treatment of Acute Coronary Syndrome and after coronary stent implantation. Clopidogrel is a pro-drug, 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 wish to 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 (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 utilized ADP as an agonist (Angiolillo et al., 2005; Angiolillo et al., 2006; Angiolillo et al., 2007; Geisler et al., 2007; Mangiacapra et al., 2010). Examination of responses to the primary activator of platelets, thrombin, is important, as it provides 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

Materials: Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). PAR1-agonist peptide (PAR1-AP; SFLLRN) and PAR4-AP (AYPGKF) were purchased from GL Biochem (Shanghai, China). FITC-PAC1 and PE-CD62P (P-selectin) were purchased from BD Pharmingen (San Jose, CA). 2-Methylthioadenosine 5′-Monophosphate Triethylammonium Salt (2-methylthio-AMP) was purchased from Sigma. Fura-2-AM and probenecid was 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 re-suspended in Tyrode’s buffer as previously described (Holinstat et al., 2006; Holinstat et al., 2007; Holinstat et al., 2012; Holinstat et al., 2013).

Surface expression of P-selectin and αIIbβ3 activation: P-selectin expression (PE-CD62P binding) and αIIbβ3 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 μM), or PAR4-AP (100 and 200 μM). Prior to stimulation, samples were incubated for 5 minutes with 50 μM of 2-methylthio-AMP or were untreated. Dose response curves (Figure1A) 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 2×10⁸ platelets/ml. PRP was incubated with Fura-2-AM (4 μM) for 60 min at 37°C. Subsequently, platelets were pelleted at 700xg for 10 min and resuspended in Tyrode’s containing 2.5 mM probenecid. Prior to stimulation, samples were incubated for 5 min with 50 μM of 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 340/510nm in a Varian fluorometer (Palo Alto, CA) for 10 min. The data are analyzed as the increase in fluorescence with agonist relative to an unstimulated control. Data is expressed as maximal Ca^{2+} mobilization and area under the curve (AUC).

**Statistics:** Demographics and medical history data was summarized in terms of median and IQR 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–Chi square test for categorical variables. Multivariable linear regression models, adjusted by age, gender, BMI and medications, were fit to access the association of diabetes and outcomes of PAC1, or P-selectin. The nonlinear association 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), * denotes P-value < 0.05, ** denotes a P-Value < 0.01 and *** denotes a P-value < 0.001. Values are expressed as mean and 95% confidence interval in fold stimulation graphs and mean ± SEM in percent inhibition graphs.
Results

Table I shows the demographics of the 141 recruited subjects stratified according to disease state. Table II shows the demographics stratified according to disease state and 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 such that partial and full platelet activation was obtained (Holinstat et al., 2006; Holinstat et al., 2007). We chose a concentration of 50 μM of 2-methylthio-AMP as this provided maximal inhibition of thrombin-mediated P-selectin expression and αIIbβ3 activation in human platelets (Figure 1, Panel A).

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 αIIbβ3 activation. To ensure that an off target effect of 2-methylthio-AMP on P2Y1 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 Figure 1B and 1C. These data indicate that any observed inhibition of P-selectin expression and αIIbβ3 activation by 2-methylthio-AMP can’t be accounted for by altered Ca2+ mobilization and therefore is not likely due to an off-target effect on P2Y1. Similar results were reported by Li et al. (Xiang et al., 2012) who demonstrate that 2-methylthio-AMP failed to inhibit Ca2+ mobilization in P2Y12 deficient mouse platelets and did not raise cAMP or induce VASP phosphorylation in wild type platelets, demonstrating that 2-methylthio-AMP inhibits platelet function through a P2Y12-dependent mechanism.

Effects of direct P2Y12- inhibition on αIIbβ3 activation in thrombin stimulated platelets.

αIIbβ3 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 (Figure 2A, 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 αIIbβ3 activation by thrombin. In these subjects, fold increase was reduced from 8.5 ± 0.6 to 5.3 ± 0.5 (27.3 +/- 6.9% inhibition, Figure 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 T2DM-patients was more evident when platelets were stimulated with high thrombin (10nM), 13.0 ± 0.7 to 7.3 ± 0.4 fold increase (40.8% inhibition, Figure 2B) in healthy subjects and 10.8 ± 0.7 to 8.3 ± 0.6 fold increase (20.3% inhibition, Figure 2B) in T2DM-subjects. This significant difference in 2-methylthio-AMP-mediated inhibition of αIIbβ3 activation was also observed with high dose PAR1-AP, but not with low dose PAR1-AP (Figure 2D), and with both high and low PAR4-AP-stimulated platelets (Figure 2F).

The large percentage of African-American-subjects enrolled permitted sub-group analysis to examine effects of P2Y12-inhibition in African-American-T2DM-subjects, which has been largely unstudied. While platelets from Caucasian-T2DM-subjects (compared to 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 (Figure 3). 2-methylthio-AMP inhibited low dose thrombin-stimulated αIIbβ3 activation in platelets from Caucasian subjects by 54.9%, compared to 9.0% in platelets from T2DM-Caucasian-subjects (Figure 3B), a significant resistance to P2Y12 antagonism. In contrast, 2-methylthio-AMP inhibited low dose thrombin-stimulated αIIbβ3 activation in platelets from African-American subjects by 38.9%, while platelets from African-American-T2DM subjects were inhibited by 43.6% (Figure 3B). Similar results were obtained when platelets were stimulated with PAR1-AP or PAR4-AP (Figure 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 (Figure 5). Results paralleled those observed with the αIIbβ3 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 +/- 2.8% inhibition vs. 28.4% +/- 5.5%, respectively, Figure 5A). With a maximal dose of thrombin (10 nM)
greater inhibition with 2-methylthio-AMP was again observed in platelets from healthy subjects compared to T2DM-subjects (31.8 +/- 2.3% vs. 16.8 +/- 3.0%, Figure 5A). Similar results were obtained when platelets were stimulated with PAR1-AP and PAR4-AP (Figure 5B and C).

Similar to αIIbβ3 activation, PAR-mediated P-selectin surface expression on platelets from Caucasian-T2DM-subjects was resistant to inhibition with 2-methylthio-AMP when compared to Caucasian healthy subjects (Figure 6, and Figure 7A and B, respectively). On the other hand, platelets from African-American-T2DM-subjects respond to 2-methylthio-AMP with % inhibition values similar to those observed in healthy African-American subjects (Figure 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 could have potential effects on platelet function (Table II). Therefore, we performed a 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 αIIbβ3 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 (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. Body mass index (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.
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 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; Angiolillo et al., 2006; Angiolillo et al., 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 physiological activator 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 αIIbβ3-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 while on clopidogrel 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; Hochholzer et al., 2006; Lev et al., 2006; Cuisset et al., 2007). Pharmacokinetic studies focus on metabolism of the pro-drug 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 up-regulation 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 due 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-methythio-AMP is due 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 it's 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 that are on P2Y12-inhibitors. Future studies will specifically address the influence of Coronary Artery Disease (CAD) and if African-American-T2DM-subjects display normal inhibition of thrombin-mediated responses with oral P2Y12-inhibitors. One study has demonstrated that 2-methythio-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 has been reported that...
2-methythio-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 Figure 1), which we believe is due 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 $\alpha_{\text{IIb}}$β3 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 Trial (1982), it was observed that Caucasians had a greater blood pressure lowering effect with beta-blockers compared to African-American-patients, while African-Americans had a better response to diuretics compared to Caucasians. These differences in race in antihypertensive response have guided therapy based on race (Messerli and Ventura, 1985; Zing et al., 1991) and have influenced guidelines for treatment (1988). Pharmacogenetic contributions are responsible for some of the racial differences to cardiovascular drug response which has the potential to further personalize 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 anti-platelet therapeutics in an African-American population. In fact, out of the 8829 samples collected as part of the International Clopidogrel Platelet Consortium, which is comprised of 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 (personal communication from Alan Shuldiner to JHC). Currently there are two trials specifically investigating anti-platelet response in African-American populations. There is a pharmacodynamic study with ticagrelor in African-American patients with stable coronary artery disease (ClinicalTrials.gov Identifier: NCT01523392) that has been completed, but is not yet published. In addition, the African-American Pharmacogenetics (ClinicalTrials.gov Identifier: NCT014081210) is a prospective genetic and platelet reactivity cohort study of African-Americans and Caucasians undergoing PCI who have received either clopidogrel or prasugrel that will be completed in June of 2014.

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 to the general public, African-Americans are disproportionately affected and have a higher incidence of T2DM (Center for Disease Control and Prevention, 2010). The differences observed between the 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 can lay the groundwork for determining the genetic basis of protection from resistance in African-American diabetics as well as for future larger clinical-outcome studies in African-Americans undergoing anti-platelet 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

Performed data analysis: Cleator, Duvernay, Colowick, Song, Harrell

Wrote or contributed to the writing of the manuscript: Cleator, Duvernay, Hamm
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Cusack NJ and Hourani SM (1982) Specific but noncompetitive inhibition by 2-alkylthio analogues of adenosine 5'-monophosphate and adenosine 5'-triphosphate of human platelet aggregation induced by adenosine 5'-diphosphate. *British journal of pharmacology* **75**:397-400.


Footnotes

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b) Presented at the American Heart Association meeting in 2011, Abstract 11336: “Racial Differences in Resistance to P2Y12 Receptor Antagonists in Thrombin-Stimulated Diabetic Platelets” John H Cleator; Matthew T Duvernay; Michael Holinstat; Nancy E Colowick; Dana T Blakemore; Frank E Harrell; Heidi E Hamm.

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d) JHC and MTD contributed equally.
Figure Legends

Figure 1. Panel A. Dose response of 2-methylthio-AMP Inhibition of thrombin-mediated P-selectin expression and αIIbβ3 activation in human platelets. Washed human platelets were stimulated with 10nM thrombin with increasing concentrations of 2-methylthio-AMP as shown. P-selectin and αIIbβ3 activation were measured as described in methods. Data are expressed as the percent max response relative to vehicle treated control. n=3. Panel B and C. 2-methylthio-AMP (50 μM) does not affect Thrombin, PAR1-AP, PAR4-AP, or ADP-mediated intracellular Ca²⁺ mobilization in human platelets. Human platelets were incubated with 50 μM of 2-methylthio-AMP or vehicle for 5 min and agonist was added as indicated (PAR1-AP = 20 μM, PAR4-AP= 200 μM and ADP= 20 μM). Ca²⁺ mobilization was measured as indicated in methods. In panel B, the data are expressed as peak Ca²⁺ response in fold increase in fluorescence relative to baseline. In panel C, the data are expressed as the area under the curve (AUC). In Panel B and C, n= 5.

Figure 2. Inhibition of agonist-mediated αIIbβ3 activation in platelets by 2-methylthio-AMP. Panel A (thrombin) is a scatter plot depicting fold stimulation of αIIbβ3 of platelets stimulated by thrombin from all subjects grouped together according to T2DM status. Panel B (Thrombin) depicts percent inhibition by 2-methylthio-AMP of platelets stimulated with thrombin plotted on the Y-axis with mean and SEM shown. Low and high dose represents 2nM and 10 nM Thrombin respectively. Panel C (PAR1-agonist peptide) is a scatter plot depicting fold stimulation of αIIbβ3 activation of platelets stimulated with PAR1-agonist peptide. Panel D (PAR1-agonist peptide) depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR1-agonist peptide plotted on the Y-axis with mean and SEM shown. Low and high dose represents 2.5 and 20 μM PAR1-agonist peptide respectively. Panel E (PAR4-agonist peptide) is a scatter plot depicting fold stimulation of αIIbβ3 activation of platelets stimulated by PAR4-agonist peptide. Panel F (PAR4-agonist peptide) depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-agonist peptide with mean and SEM shown. Low and high dose represents 100 and 200 μM PAR4-agonist peptide respectively.

Figure 3. Racial differences in inhibition of thrombin-mediated αIIbβ3 activation in platelets by 2-methylthio-AMP. Panel A is a scatter plot depicting fold stimulation of αIIbβ3 activation of platelets stimulated with thrombin from subjects stratified on basis of racial background as well as T2DM status. Panel B depicts
percent inhibition with 2-methylthio-AMP from subjects stratified on basis of race and T2DM status plotted on the Y-axis with mean and SEM shown. Low and high dose represents 2nM and 10 nM Thrombin respectively. AA=African-American.

**Figure 4. Panel A. Racial differences in inhibition of PAR1-agonist peptide-mediated αIIbβ3 activation in platelets by 2-methylthio-AMP.** Figure depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR1-agonist peptide from subjects stratified on basis of race and T2DM status plotted on the Y-axis with mean and SEM shown. Low and high dose represents 2.5 and 20 μM PAR1-agonist peptide respectively. **Panel B. Racial differences in inhibition of PAR4-agonist peptide-mediated αIIbβ3 activation in platelets by 2-methylthio-AMP.** Figure depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-agonist peptide from subjects stratified on basis of race and T2DM status plotted on the Y-axis with mean and SEM shown. Low and high dose represents 100 and 200 μM PAR4-agonist peptide respectively. AA=African-American.

**Figure 5. Inhibition of agonist-mediated P-selectin activation in platelets by 2-Methylthio-AMP. Panel A (thrombin) depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with thrombin from subjects stratified on the basis of T2DM status with mean and SEM shown. Low and high dose represents 2nM and 10 nM Thrombin respectively. Panel B (PAR1-agonist peptide) depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR1-agonist peptide from subjects stratified on basis of T2DM status plotted on the Y-axis with mean and SEM shown. Low and high dose represents 2.5 and 20 μM PAR1-agonist peptide respectively. Panel C (PAR4-agonist peptide) depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-agonist peptide from subjects stratified on basis of T2DM status with mean and SEM shown. Low and high dose represents 100 and 200 μM PAR4-agonist peptide respectively.

**Figure 6. Racial differences in inhibition of thrombin-mediated P-selectin activation in platelets by 2-methylthio-AMP.** Figure depicts 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 SEM shown. Low and high dose represents 2nM and 10 nM Thrombin respectively. AA=African-American.
Figure 7. Racial differences in inhibition of PAR1-agonist peptide-mediated P-selectin activation in platelets by 2-methylthio-AMP. Panel A depicts percent inhibition with 2-methylthio-AMP of platelets stimulated by PAR1-agonist peptide from subjects stratified on basis of T2DM status and race and with mean and SEM shown. Low and high dose represents 2.5 and 20 μM PAR1-agonist peptide respectively. Panel B. Racial differences in inhibition of PAR4-agonist peptide-mediated P-selectin activation in platelets by 2-methylthio-AMP. Figure depicts percent inhibition with 2-methylthio-AMP of platelets stimulated with PAR4-agonist peptide from subjects stratified on basis of T2DM status and race and with mean and SEM shown. Low and high dose represents 100 and 200 μM PAR4-agonist peptide respectively. AA=African-American.
### Table I. Demographics of healthy and T2DM subjects recruited

<table>
<thead>
<tr>
<th>Clinical Characteristics (n=141)</th>
<th>healthy (n=75)</th>
<th>T2DM (n=66)</th>
<th>Test Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29 44 55</td>
<td>46 54 60</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>0.873</td>
</tr>
<tr>
<td>BMI</td>
<td>22.7 25.8 30.5</td>
<td>28.0 32.5 37.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mmHG)</td>
<td>113.0 120.0 132.0†</td>
<td>120.0 126.0 137.5‡</td>
<td>0.092</td>
</tr>
<tr>
<td>DBP (mmHG)</td>
<td>70.0 78.5 83.0†</td>
<td>70.0 78.0 86.0§</td>
<td>0.860</td>
</tr>
<tr>
<td>HgA1C (%)</td>
<td>ND</td>
<td>6.40 6.90 8.25 NA</td>
<td></td>
</tr>
<tr>
<td>Smokers (n, %)</td>
<td>2 (2.7%)</td>
<td>4 (6.1%)</td>
<td>0.319</td>
</tr>
<tr>
<td>Medications (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>4 (5.3%)</td>
<td>25 (37.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>4 (5.3%)</td>
<td>23 (34.8%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACE Inhibitor</td>
<td>4 (5.3%)</td>
<td>24 (36.4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ARB</td>
<td>3 (4.0%)</td>
<td>7 (10.6%)</td>
<td>0.127</td>
</tr>
<tr>
<td>Calcium Channel Blocker</td>
<td>1 (1.3%)</td>
<td>10 (15.2%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Statin</td>
<td>8 (10.7%)</td>
<td>35 (53.0%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Demographics of healthy and T2DM subjects enrolled. § The one subject in the healthy group on metformin is taking this for treatment of polycystic ovary syndrome. † = n of 26, ‡ = n of 31. Test statistic was performed using the Wilcoxon rank sum test for continuous variables and Pearson –Chi square test for categorical variables. In some cases blood pressure readings were taken from the Electronic Medical Record within a 6 month time frame from time of blood draw. Abbreviations: T2DM= Type 2 Diabetes Mellitus, SBP= systolic blood pressure, DBP= diastolic blood pressure, Statin=HMG-CoA reductase inhibitors, ACE=Angiotensin-Converting Enzyme, ARB=Angiotensin II Receptor Blocker, ASA= aspirin. a b c represents the lower quartile a, the median b, and the upper quartile c.
Table II. Demographics of healthy subjects compared to 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 (years)'</td>
<td>28 46 58</td>
<td>52 55 62</td>
<td>0.007</td>
<td>30 42.5 50</td>
<td>45 51 59</td>
<td>0.003</td>
</tr>
<tr>
<td>Female (n, %)</td>
<td>29 (59.2%)</td>
<td>14 (45.2%)</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>0</td>
<td>2 (5.7%)</td>
<td>0.215</td>
</tr>
<tr>
<td>BMI '</td>
<td>22.5 25.1 29.3</td>
<td>28.8 33.2 40.8</td>
<td>&lt;0.001</td>
<td>24.1 25.8 31.1</td>
<td>28.1 32.2 36.7</td>
<td>0.002</td>
</tr>
<tr>
<td>SBP (mmHG)'</td>
<td>112.5 120.0 125.5*</td>
<td>120 123.0 135†</td>
<td>0.125</td>
<td>122 132 136.5‡</td>
<td>125 134 139.5§</td>
<td>0.524</td>
</tr>
<tr>
<td>DBP (mmHG)'</td>
<td>70 79 84*</td>
<td>70 79 86†</td>
<td>0.971</td>
<td>69.5 72 79‡</td>
<td>69 75 85.5§</td>
<td>0.724</td>
</tr>
<tr>
<td>HgA1C (%)'</td>
<td>ND</td>
<td>6.3 6.7 7.3</td>
<td>NA</td>
<td>ND</td>
<td>6.8 7.4 10.6</td>
<td>NA</td>
</tr>
<tr>
<td>Smokers</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></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</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>
<tr>
<td>β-Blocker</td>
<td>2 (4.1%)</td>
<td>11 (35.5%)</td>
<td>&lt;0.001</td>
<td>2 (7.7%)</td>
<td>12 (34.3%)</td>
<td>0.015</td>
</tr>
<tr>
<td>ACE Inhibitor</td>
<td>3 (6.1%)</td>
<td>11 (35.5%)</td>
<td>&lt;0.001</td>
<td>1 (3.8%)</td>
<td>13 (37.1%)</td>
<td>0.002</td>
</tr>
<tr>
<td>ARB</td>
<td>2 (4.1%)</td>
<td>3 (9.7%)</td>
<td>0.314</td>
<td>1 (3.8 %)</td>
<td>4 (11.4%)</td>
<td>0.286</td>
</tr>
<tr>
<td>CCB</td>
<td>0</td>
<td>4 (12.9%)</td>
<td>0.01</td>
<td>1 (3.8%)</td>
<td>6 (17.1%)</td>
<td>0.107</td>
</tr>
</tbody>
</table>
### Clinical Characteristics

<table>
<thead>
<tr>
<th></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><strong>Diuretic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (6.1%)</td>
<td>7 (22.6%)</td>
<td>0.03</td>
<td>3 (11.5%)</td>
<td>14 (40.0%)</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>Nitrates</strong></td>
<td>0</td>
<td>2 (6.5%)</td>
<td>0.072</td>
<td>0</td>
<td>3 (8.6%)</td>
<td>0.126</td>
</tr>
<tr>
<td><strong>Metformin</strong></td>
<td>0</td>
<td>23 (74.2%)</td>
<td>&lt;0.001</td>
<td>1 (3.8%)</td>
<td>19 (54.3%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td>0</td>
<td>7 (22.6%)</td>
<td>&lt;0.001</td>
<td>0</td>
<td>13 (37.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Sulfonylurea</strong></td>
<td>0</td>
<td>5 (16.1%)</td>
<td>0.004</td>
<td>0</td>
<td>8 (22.9%)</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Glitazone</strong></td>
<td>0</td>
<td>3 (9.7%)</td>
<td>0.026</td>
<td>0</td>
<td>1 (2.9%)</td>
<td>0.385</td>
</tr>
<tr>
<td><strong>Gliptin</strong></td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>0</td>
<td>4 (11.4%)</td>
<td>0.075</td>
</tr>
<tr>
<td><strong>Incretin mimetic</strong></td>
<td>0</td>
<td>4 (12.9%)</td>
<td>0.01</td>
<td>0</td>
<td>1 (2.9%)</td>
<td>0.385</td>
</tr>
<tr>
<td><strong>Warfarin</strong></td>
<td>0</td>
<td>2 (6.5%)</td>
<td>0.072</td>
<td>0</td>
<td>3 (8.6%)</td>
<td>0.126</td>
</tr>
<tr>
<td><strong>NSAID</strong></td>
<td>2 (4.1%)</td>
<td>4 (12.9%)</td>
<td>0.144</td>
<td>1 (3.8%)</td>
<td>3 (8.6%)</td>
<td>0.461</td>
</tr>
<tr>
<td><strong>Acetaminophen</strong></td>
<td>1 (2.0%)</td>
<td>3 (9.7%)</td>
<td>0.127</td>
<td>1 (3.8%)</td>
<td>5 (14.3%)</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Abbreviations: T2DM= Type 2 Diabetes Mellitus, SBP= systolic blood pressure, DBP= diastolic blood pressure, Cauc=Caucasian, AA=African-American, Statin=HMG-CoA reductase inhibitors, ACE=Angiotensin-Converting Enzyme, ARB=Angiotensin II Receptor Blocker, ASA= aspirin. Test statistic was performed using the Wilcoxon rank sum test for continuous variables and Pearson –Chi square test for categorical variables.

* = n of 21, † = n of 23, ‡ = n of 5, § = n of 8. In some cases blood pressure readings were taken from the Electronic Medical Record and were obtained within a 6 month time frame from time of blood draw.

a, b, c, represents the lower quartile a, the median b, and the upper quartile c.
Abbreviations: N= number, NA= not applicable, ND= no data, SBP= systolic blood pressure, DBP= diastolic blood pressure, T2DM= Type 2 Diabetes Mellitus, Cauc= Caucasian, AA= African-American, ACE= Angiotensin-Converting Enzyme, Statin= HMG-CoA reductase inhibitors, ARB= Angiotensin II Receptor Blocker, ASA= aspirin
Figure 1

A. % Max response

- P-Selectin Expression
- α.Ibbβ3 Activation

log [2-methylthio-AMP]

B. Peak Ca\(^{2+}\) fluorescence (fold increase relative to untreated)

- Control
- 2-methylthio-AMP

Thrombin, PAR1-AP, PAR4-AP, ADP

C. Total Ca\(^{2+}\) fluorescence (AUC)

- Control
- 2-methylthio-AMP

Thrombin, PAR1-AP, PAR4-AP, ADP
Figure 2

A. 2 nM Thrombin vs. 10 nM Thrombin

B. % Inhibition with 2-methylthio-AMP

C. 2.5 μM PAR1-AP vs. 20 μM PAR1-AP

D. % Inhibition with 2-methylthio-AMP

E. 100 μM PAR4-AP vs. 200 μM PAR4-AP

F. % Inhibition with 2-methylthio-AMP
Figure 4

A. 2.5 μM PAR1-AP

% Inhibition with 2-methylthio-AMP (μl/l3 activation)

Cauc Non-T2DM  |  Cau T2DM  |  AA Non-T2DM  |  AA T2DM  

B. 100 μM PAR4-AP

% Inhibition with 2-methylthio-AMP (μl/l3 activation)

Cauc Non-T2DM  |  Cau T2DM  |  AA Non-T2DM  |  AA T2DM  

Cauc T2DM  |  AA Non-T2DM  |  AA T2DM
Racial differences in resistance to P2Y12 receptor antagonists in Type-2 diabetic subjects
John H. Cleator MD, PhD, Matthew T. Duvernay PhD, Michael Holinstat PhD, Nancy E. Colowick BA, Med, Willie J. Hudson MS, Yanna Song MS, Frank E. Harrell PhD, Heidi E. Hamm PhD
Journal of Pharmacology and Experimental Therapeutics

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.