Mechanism of Race-Dependent Platelet Activation Through the Protease-Activated Receptor-4 and Gq Signaling Axis

Benjamin E. Tourdot, Stanley Conaway, Katrin Niisuke, Leonard C. Edelstein, Paul F. Bray, Michael Holinstat

**Objective**—Black individuals are at an increased risk of myocardial infarction and stroke, 2 vascular diseases with strong thrombotic components. Platelet activation is a key step in platelet clot formation leading to myocardial infarction and stroke, and recent work supports a racial difference in platelet aggregation through the thrombin protease-activated receptors (PARs). The underlying mechanism for this racial difference, however, has not been established. Determining where in the signaling cascade these racial differences emerge will aid in understanding why individuals of differing racial ancestry may possess an inherent difference in their responsiveness to antiplatelet therapies.

**Approach and Results**—Washed human platelets from black volunteers were hyperaggregable in response to PAR4-activating peptide compared with whites. Interestingly, the racial difference in PAR4-mediated platelet aggregation persisted in platelets treated ex vivo with aspirin and 2MeSAMP (2-methylthioadenosine 5′-monophosphate triethylammonium salt hydrate), suggesting that the racial difference is independent of secondary feedback. Furthermore, stimulation of platelets from black donors with PAR4-activating peptide showed a potentiated level of activation through the Gq pathway compared with platelets from white donors. Differences in signaling included increased Ca2+ mobilization, Rap1 (Ras-related protein 1) activation, and integrin αIIbβ3 activation with no observed difference in platelet protein expression between the groups tested.

**Conclusions**—Our study is the first to demonstrate that the Gq pathway is differentially regulated by race after PAR4 stimulation in human platelets. Furthermore, the racial difference in PAR4-mediated platelet aggregation persisted in the presence of cyclooxygenase and P2Y12 receptor dual inhibition, suggesting that current antiplatelet therapy may provide less protection to blacks than whites. (Arterioscler Thromb Vasc Biol. 2014;34:2644-2650.)

**Key Words:** blood platelet continental population groups protease-activated receptor 4 thrombin thrombosis

Platelet activation is a key step in the formation of an occlusive thrombus. Thrombotic events precipitated by vascular disease can lead to stroke or myocardial infarction (MI), 2 of the leading causes of death in the United States. Furthermore, MI and stroke disproportionately burden black individuals relative to white individuals. Because of the importance of thrombosis in the pathogenesis of MI and stroke, the activation of platelets from healthy black or white donors in response to a panel of common platelet agonists was tested recently by our group. Interestingly, signaling through 1 of the 2 thrombin receptors expressed on the human platelet, protease-activated receptor-4 (PAR4), resulted in greater aggregation in platelets from black volunteers as compared with white volunteers.

See accompanying editorial on page 2524

Thrombin is a potent platelet agonist whose regulation of platelet function has been studied extensively. Thrombin initiates platelet activation through the enzymatic cleavage of the N-terminal extracellular domain of the PARs. This new N-terminus serves as a tethered ligand that activates PAR G-protein–coupled receptor signaling. Human platelets express 2 PARs, PAR1 and PAR4, that signal through both overlapping and unique biochemical steps. Specifically, the stimulation of PARs leads to the activation of the G12/13 and Gq pathways. The initiation of Gq signaling leads to a rise in intracellular Ca2+, which activates Ras-related protein 1 (Rap1), culminating in the activation of αIIbβ3, a surface receptor required for platelet aggregation. Although this pathway has been studied extensively, the component(s) that may be differentially regulated by race remains unclear. The current study sought to determine how PAR4 reactivity is differentially regulated between healthy black and white donors. In the presence of PAR1 antagonist, platelets from black donors are hyper-responsive to thrombin compared with platelets from white donors. As PAR1 antagonists have been approved for use in the clinic, understanding the differential
signaling elicited by thrombin activation through PAR4 will have important clinical implications. In this study, we show that racial differences in the PAR4 Gq signaling pathway including Ca\(^{2+}\) mobilization, Rap1 activation, and α\(\text{IIb}\)β\(\text{3}\) activation contribute to the racial difference observed in PAR4-mediated platelet aggregation. Understanding these differences that occur in the PAR4 pathway in platelets may lead to targeted therapies to reduce thrombotic risk in black individuals.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

### Results

**PAR4-Mediated Aggregation Is Differentially Regulated by Race**

Previously published work suggests that a racial component exists for PAR4-mediated platelet aggregation.\(^7\) Although that study was conducted on >150 healthy individuals, recruitment was primarily limited to a localized region (Houston, TX). To determine whether differences in PAR4-activating peptide (PAR4-AP)–mediated platelet aggregation are because of racial differences and not unique to the Southwestern United States, PAR4-AP platelet aggregation was measured in platelets from healthy black and white subjects from the Philadelphia region. Platelets from black donors in platelet-rich plasma (PRP) stimulated with PAR4-AP had an increase in maximum aggregation relative to platelets in PRP from white donors with significant differences in maximal aggregation between black and white donors being observed at 50 and 75 μmol/L PAR4-AP (Figure 1A and 1B). No difference in PAR4-AP–induced aggregation was observed at higher concentrations of PAR4-AP (100 μmol/L). In addition to platelets, PRP contains other plasma constituents, which may be differentially expressed by race and indirectly influence platelet activity. No difference in PAR4-AP-mediated aggregation was observed at higher concentrations of PAR4-AP (100 μmol/L). In addition to platelets, PRP contains other plasma constituents, which may be differentially expressed by race and indirectly influence platelet activity. No difference in PAR4-AP-mediated aggregation was observed when washed platelets from white donors were resuspended in platelet poor plasma from black donors or vice versa (Figure 1C and 1D). These data suggest that the components of plasma are not a major contributor to the racial difference in PAR4-mediated platelet aggregation.

To eliminate potential confounding factors in the plasma, platelets were isolated from the plasma by centrifugation and the activation of washed platelets was measured in response to stimulation with thrombin, PAR1-AP or PAR4-AP. Consistent with platelet responses observed in PRP (Figure 1A and 1B), the maximal aggregation of washed platelets from black and white donors stimulated with thrombin (Figure 1A in the online-only Data Supplement) or PAR1-AP (Figure 1B in the online-only Data Supplement) was the same but enhanced in PAR4-AP–stimulated platelets from black donors compared with white donors with significant differences in aggregation by race being observed with as little as 10 μmol/L PAR4-AP (Figure 1E–1H). Maximal platelet aggregation and time to 50% aggregation in response to PAR4-AP were evaluated in platelets from blacks (Figure 1G and 1H, respectively), both of which demonstrated that platelets from black donors were hyperaggregable compared with those from white donors in response to PAR4-AP. Washed platelets were observed to be more sensitive to PAR4-AP stimulation compared with platelets in PRP, possibly because of nonspecific binding of the agonist peptide.

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**Nonstandard Abbreviations and Acronyms**

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<td>activating peptide</td>
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<td>protease-activated receptor</td>
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**Figure 1.** Platelets from blacks are hyperaggregable to protease-activated receptor-4-activating peptide (PAR4-AP) stimulation, as compared with whites. A, Representative aggregation tracings of platelets in platelet-rich plasma (PRP) from a white or black donor in response to 75 μmol/L PAR4-AP. B, Quantification of max aggregation of black (n=8) or white (n=8) platelets in PRP in response to an increasing concentration of PAR4-AP. C, Washed platelets from white donors were resuspended in their own platelet poor plasma (PPP) or PPP from a black donor than stimulated with increasing concentrations of PAR4-AP. D, The reciprocal experiment from C was performed with washed platelets from black donors. Representative aggregation tracings of washed platelets from black and white donors in response to 25 μmol/L (E) or 50 μmol/L (F) of PAR4-AP. The aggregation of washed platelets from black and white donors in response to increasing concentrations of PAR4-AP was evaluated by both maximum aggregation (G; n=14–22) and time to 50% aggregation (H; n=5–10). Data represent mean±SEM. Data were analyzed using a 2-way ANOVA.
to other plasma proteins. To determine whether potential differences exist in the kinetics of platelet activation, the time to 50% aggregation was measured, which encompasses both the lag time (the time from agonist addition until the start of aggregation) as well as the rate of aggregation. Because not all donors aggregated in response to threshold doses of PAR4-AP, platelets from subjects who failed to reach 50% aggregation were eliminated from the analysis. For the donors who did not reach 50% aggregation, 6 of 13 white donors at 35 μmol/L of PAR4-AP and 3 of 12 white donors at 50 μmol/L of PAR4-AP were excluded from the analysis. Even with the absence of several white subjects because of lack of platelet aggregation, platelets from white donors were slower to 50% aggregation compared with platelets from black donors at low concentrations of PAR4-AP (35–50 μmol/L; Figure 1H). These data suggest that the racial difference in PAR4-mediated platelet activation is intrinsic to the platelet.

Racial Differences in PAR4-Mediated Platelet Activation Are Independent of Cyclooxygenase and P2Y12

Platelet aggregation occurs in 2 phases in response to weak stimulation such as low concentrations of PAR4-AP. Weak agonists induce the primary phase of aggregation, which then incites the generation or release of soluble platelet agonists such as ADP and arachidonic acid, which are capable of eliciting a secondary phase of aggregation.14,18,19 Although no racial differences in platelet aggregation were found with ADP and arachidonic acid, a precursor to thromboxane, these tests were performed with only a single concentration of each agonist. It is therefore possible that racial differences occur at other concentrations of ADP and arachidonic acid, which were not tested in previous studies.7 To determine whether racial differences in PAR4-AP–mediated platelet aggregation were because of differences in secondary feedback signaling through ADP or thromboxane, platelets were stimulated with PAR4-AP in the presence of aspirin, a cyclooxygenase inhibitor that blocks thromboxane production, and 2MeSAMP (2-methylthioadenosine 5′-monophosphate triethylammonium salt hydrate), a P2Y12 (ADP receptor) antagonist. Platelets from blacks remained hyperaggregable to PAR4-AP stimulation compared with whites when treated ex vivo with 2MeSAMP (Figure 2A), aspirin (Figure 2B), or both (Figure 2C; Figure II in the online-only Data Supplement). Although dual inhibition seemed to have a synergistic inhibitory effect on platelet aggregation, the persistence of racial differences in platelets stimulated with PAR4-AP in the presence of dual platelet inhibitors suggests that these differences are at least partially independent of thromboxane and ADP signaling. In addition, to assess whether there was a racial difference in thromboxane receptor–induced platelet aggregation, washed platelets from black or white donors were stimulated with increasing concentrations of the thromboxane mimetic, U46619. There was no racial difference in maximal platelet aggregation in response to U46619 (Figure IC in the online-only Data Supplement).

PAR4 Surface Expression

Platelets express appreciable amounts of PAR1 and PAR4 on their surface.10 One possible explanation for the racial difference in PAR4-mediated platelet aggregation is an increase in the surface expression of PAR4 on platelets from black donors relative to platelets from white donors. To determine whether this was the case, surface expression of PAR4 was measured in platelets from white and black donors. Platelets from black donors had higher PAR4 expression compared with white donors (Figure 3A and 3B).

Figure 2. The racial difference in protease-activated receptor-4 (PAR4)–mediated platelet activation persists in the presence of ex vivo cyclooxygenase and P2Y12 inhibition. Washed platelets from black (n=8) or white (n=8) donors were pretreated with either 2MeSAMP (2-methylthioadenosine 5′-monophosphate triethylammonium salt hydrate; A) for 20 minutes, aspirin (B) for 40 minutes, or both (C) before PAR4-AP stimulation. Each figure contains a representative tracing of platelets from a black and white donor at the concentration where the racial difference is maximized. Data represent mean±SEM. Data were analyzed using a 2-way ANOVA.

Figure 3. Protease-activated receptor-4 (PAR4) surface expression is same between platelets from blacks and whites. PAR4 surface expression of resting platelets from white and black donors was measured by flow cytometry. A, A representative histogram of the PAR4 surface expression on platelets from a black or white donor. B, Graphs depicting the PAR4 surface expression of blacks (n=5) and whites (n=6). Data represent mean±SEM (2-tailed t test).
Ca²⁺ Mobilization Is Differentially Regulated by Race Through PAR4

Because no racial difference was observed in the surface expression of PAR4, the activation status of key biochemical components of the PAR4 pathway was assessed in platelets from blacks and whites. PAR4 stimulation of platelets leads to a rise in intracellular Ca²⁺, which is critical for some of the key steps leading to normal PAR4-mediated platelet aggregation including protein kinase C (PKC), Rap1, and granule secretion. Platelets from black donors stimulated with PAR4-AP (50 μmol/L) had a significantly higher increase in maximal Ca²⁺ mobilization compared with platelets from white donors (Figure 4A and 4B).

Rap1 and PKC Activity Are Differentially Regulated by Race

Rap1 and PKC are regulated in part by Ca²⁺ mobilization. Because Ca²⁺ mobilization was potentiated in platelets from black donors (Figure 4), it was important to identify whether the difference in free Ca²⁺ in the platelet after PAR4-AP translates to a difference in activation of these key biochemical intermediates in platelet aggregation. Rap1, a small G-protein, is known to be important for inside-out activation of αIIbβ3, an essential step in platelet aggregation. Rap1 is activated as part of the PAR4 Gq pathway downstream of Ca²⁺ mobilization. To determine whether the racial difference in Ca²⁺ mobilization leads to a difference in Rap1 activation between platelets from white or black donors stimulated with PAR4-AP, Rap1 activity was measured over time after stimulation. Rap1 activation was elevated in platelets from black donors at each time point measured and was significantly higher in platelets from black donors at 5 minutes after stimulation with 50 μmol/L PAR4-AP (Figure 5A and 5B).

PKC activation is a key intermediate step downstream of PAR4 Gq signaling in the platelet. Ca²⁺ is known to be essential for the activation of several PKC isoforms involved in platelet function. Therefore, to determine whether PKC was differentially regulated by race, PKC activation was measured by its ability to phosphorylate Pleckstrin, a major PKC substrate in platelets, after stimulation by PAR4-AP. After stimulation with PAR4-AP, platelets from black donors showed a significantly potentiated phosphorylation of Pleckstrin compared with platelets from whites at 30 seconds, whereas no difference in total Pleckstrin levels was observed between donors (Figure 5C and 5D). Taken together, a measurable difference was observed in the level of platelet function through the PAR4 pathway in platelets from black and white donors. Specifically, the data suggest that a racial difference in platelet Ca²⁺ mobilization and its downstream effectors, Rap1 and PKC, play at least a partial role in mediating these differential effects (Figures 4 and 5).
Integrin αIIbβ3, Differentially Regulated Through PAR4

Because Rap1 is known to be an essential regulator of integrin αIIbβ3 activation\(^1\) and its activity is differentially regulated by race (Figure 5), it is likely that αIIbβ3 activity is also potentiated in platelets from blacks stimulated with PAR4-AP compared with whites. To identify whether the potentiated activity observed in platelets from black donors is measurable at the level of αIIbβ3, platelets from black and white donors were stimulated with PAR4-AP in the presence of FITC-conjugated PAC-1, an antibody specific for the active conformation of αIIbβ3, and the level of active αIIbβ3 was measured on the surface of platelets by flow cytometry. On stimulation with 50 μmol/L of PAR4-AP, platelets from black donors had significantly increased levels of active αIIbβ3 compared with platelets from white donors (Figure 6A and 6B).

Potentiation of the biochemical intermediate steps in PAR4 signaling in the platelet in blacks may be because of several factors including altered receptor number, variability of the signalosome, or genetic variability in expression levels of the proteins involved in the signaling after PAR4 activation. To determine whether the increased signaling in platelets from black donors was because of a differential expression of some of the key signaling proteins in the PAR4 pathway, protein levels for Rap1 and αIIbβ3 were measured from resting platelets from black and white donors. Surprisingly, all donors were observed to have similar levels of these proteins as measured by Western blot and normalized to GAPDH (Figure 6C–6E). These data suggest that potential differences in the expression of these key intermediates are not the determining factor mediating differential platelet activity by race through the PAR4 pathway.

Discussion

Thrombotic diseases such as MI and stroke disproportionally burden blacks.\(^1,6\) Therefore, our group sought to investigate the underlying biochemical differences mediating the observed racial difference in platelet reactivity. Recently, we were able to show that platelets from black donors were hyperaggregable to PAR4 stimulation compared with platelets from white donors.\(^7\) However, the mechanism responsible for this difference in signaling remained unclear. We demonstrate here for the first time that the racial difference in PAR4-mediated platelet aggregation is because of potentiation of signaling components in the PAR4 Gq pathway of platelets from blacks compared with whites. The racial difference in PAR4-mediated platelet activation was not because of differences in the protein expression of key PAR4 signaling components including the receptor (Figure 3), the small G protein Rap1, and the integrin αIIbβ3 (Figure 6). The observation that Rap1 and αIIbβ3 are not differentially expressed by race is consistent with previous findings that platelets from blacks and whites have similar levels of aggregation to all other agonists tested except PAR4-AP.\(^7\) The magnitude because of race in the difference of activation of the PAR4 Gq pathway components tested including Ca\(^{2+}\) mobilization (Figure 4), Rap1 (Figure 5), and αIIbβ3 (Figure 6) is similar with \(\approx\)1.5- to 2-fold difference. These data are supportive of racial differences in PAR4-mediated platelet activation being propagated through differences in Ca\(^{2+}\) mobilization and signaling components upstream of Ca\(^{2+}\) that may contribute to differences in platelet function.

Our previous study, which identified a racial difference in platelet aggregation, consisted of a cohort recruited primarily from the Houston area (with a small cohort from the Philadelphia area used in thrombin studies). It is possible that...

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**Figure 6.** αIIbβ3 activation in platelets from blacks relative to whites. The activation status of αIIbβ3 in washed platelets from white and black donors stimulated with 50 μmol/L protease-activated receptor-4-activating peptide (PAR4-AP) was measured by flow cytometry. A. Representative histograms of platelets from white and black donors stimulated with PAR4-AP for 300 seconds. B. Graph representing the mean fluorescence intensity of platelets from black (n=15) or white (n=16) donors stimulated with PAR4-AP at various times. The protein expression of αIIb, Rap1, and GAPDH was measured in resting platelets from black (n=8) and white (n=10) donors by Western blot. C. Representative blot of αIIb, Rap1, and GAPDH. Graphs depicting the levels of (D) αIIb or (E) Rap1 normalized to the amount of GAPDH. Data represent mean±SEM (*P<0.05, 1-tailed t test).
the racial difference observed in PAR4-mediated platelet activation could be because of unique genetic or environmental factors in blacks and whites in that geographic region. The present study therefore sought to determine whether hyper-aggregation in platelets from blacks in PRP compared with whites was genetic or a combination of genetic and environmental. The current findings are supportive, based on the newly recruited cohort from Philadelphia that the difference in PAR4-mediated platelet aggregation is because of racial rather than regional differences. In addition, although aggregation studies using PRP are more physiologically relevant than washed platelets, the components of the plasma including lipids and proteins can confound the interpretation of potential differences inherent in platelet signaling from these groups. Hence, aggregation studies were performed with isolated washed platelets supporting that the observed racial difference in platelet aggregation are because of an inherent difference in the platelets themselves (Figure 1G and 1H).

The racial difference in PAR4-mediated platelet aggregation in washed platelets occurred at submaximal concentrations of PAR4-AP (<60 µmol/L; Figure 1G). Although the entire dose–response for aggregation to PAR4-AP was substantially shifted to the left in platelets from black donors compared with those from white donors, the largest differences in aggregation were observed in the dynamic range for PAR4-AP–mediated aggregation occurring below the EC80. As many studies have focused on high concentrations of PAR4-AP (>EC80), it is possible that potential racial differences in these platelet function studies were overlooked. Furthermore, at lower concentrations of PAR4-AP secondary agonists generated or released by platelets play a more important role in mediating full platelet aggregation.14,18,19,24

We demonstrate that a racial differences in PAR4-mediated aggregation still occur even in the presence of a cyclooxygenase inhibitor and P2Y12 antagonist, which block the production of thromboxane from arachidonic acid and ADP signaling, respectively (Figure 2). Together these data suggest that the hyperactivation of PAR4-stimulated platelets from blacks compared with those from whites is in part because of differences in the expression or activation of an effector within the PAR4 pathway.

The current study identified several biochemical intermediate steps in the PAR4 pathway, which are differentially regulated by race from mobilization of intracellular Ca2+ to αIIbβ3 activation and aggregation. The role of Ca2+ in differential signaling in the platelet by race is consistent with previous studies that reported a racial difference in Ca2+ mobilization in platelets.25 Interestingly, previous studies have also shown the kinetics of Ca2+ mobilization to be dependent on several factors including the method of activation. For example, PAR1- and PAR4-stimulated platelets show differing kinetics for Ca2+ mobilization,15 suggesting that Ca2+ release is uniquely regulated in these 2 pathways.15 In addition, studies have shown in other cell types that Ca2+ mobilization is differentially regulated by race because of differences in ion channels such as sodium and potassium transporters.26 Within platelets, Ca2+ release is regulated by a complex signaling network, including IP3R (inositol trisphosphate receptor), STIM1 (stromal interaction molecule 1), Orai1, and TRPC6 (transient receptor potential channel C6), all of which may be involved in the differential signaling observed in Ca2+ mobilization after stimulation of the PAR4 pathway in blacks and whites.27 Future work is required to determine whether the components responsible for the regulation of Ca2+ mobilization contribute to the racial difference in PAR4-mediated platelet activation. Another possibility underlying the potentiation in Ca2+ mobilization by race are differences in activation of proximal signaling components of the PAR4 pathway including the PAR4 receptor, Gq turnover, and PLCβ (phospholipase C) activation, each of which is currently under investigation in the laboratory. Finally, novel signaling components are being investigated, which may play an important role in racial differences of platelet reactivity. One such novel protein which was recently identified to be differentially expressed by race in human platelets, phosphatidylcholine transfer protein,28 is actively being investigated to determine whether differential expression of phosphatidylcholine transfer protein regulates PAR4-mediated platelet activation upstream of Ca2+ mobilization. Future investigation of a potential racial difference in relative risk for thrombosis in cardiovascular patients treated with dual antiplatelet therapy (aspirin and Plavix) will support the use of personalized treatment (based on genetic factors preserved in different racial groups) for the risk of a thrombotic events. The development of unique therapeutic approaches for the prevention of thrombosis in each of these genetically unique populations may significantly decrease the observed morbidity and mortality because of occlusive thrombus formation and stroke after platelet activation.

This is the first in-depth investigation identifying the racial differences inherent in PAR4-mediated platelet activation. Platelet reactivity in blacks is significantly elevated compared with that observed in whites even in the presence of dual platelet inhibition (P2Y12 antagonist and aspirin). Although the current study suggests that white patients on dual antiplatelet therapy may be better protected from thrombotic events compared with their black counterparts, this hypothesis will need to be confirmed in patients taking dual antiplatelet therapy (cyclooxygenase-1 and P2Y12 inhibitors). This study is timely as the PAR1 antagonist vorapaxar has recently been approved for the prevention of MI. In the presence of a PAR1 antagonist, thrombin is only able to activate platelets via PAR4. As more data on the PAR1 antagonist become available, it will be important to determine whether black patients taking PAR1 antagonists, in addition to cyclooxygenase and P2Y12 inhibitors, are equally protected from thrombotic events as white patients. Recent work including the current study suggests that platelets from black individuals are hyper-responsive to PAR4 stimulation. Thus, the development of a PAR4 antagonist may represent a targeted protection from thrombosis and MI in particular segments of the population at risk for cardiovascular diseases.

**Acknowledgments**

We thank Joanne Vesci for recruiting subjects and drawing blood. The protease-activated receptors-4 (PAR4) antibody was a gift from Dr Marvin Nieman at Case Western Reserve University.
Sources of Funding
This work was supported in part by the National Institutes of Health Grants HL114405, MD007880, and GM105671 (M. Holinstat) and HL102482 (P.F. Bray).

Disclosures
None.

References
Materials and Methods

Reagents: PAR4-activating peptide (PAR4-AP) (AYPGKF) and PAR1-AP (SFLLRN) (GL Biochem, Shanghai, China), Fluo-4AM (Life Technologies, Grand Island, NY), PAR4 antibody (a gift from Marvin Nieman) PKC-substrate antibody and PAC1-FITC (Cell signaling, Danvers, MA.), 2MeSAMP and aspirin (Sigma Aldrich St. Louis, MO), thrombin (enzyme research laboratories, South Bend, IN), U46619 (Cayman Chemical Company, Ann Arbor, MI) Pleckstrin antibody (BD Biosciences, San Jose, CA), αIIb, Rap1, and GAPDH antibodies (Santa Cruz Biotechnology, Inc, Dallas, TX), Secondary antibodies (LI-COR Biosciences, Lincoln, NE), glutathione beads (GE-Healthcare, Waukesha, WI), All other reagents were purchased from Sigma Aldrich (St. Louis, MO), unless otherwise specified.

Preparation of human platelets: The Thomas Jefferson University Institutional Review Board approved all studies involving human research. Written informed consent was obtained from all volunteers prior to blood collection. Blood was drawn from the antecubital vein of self-reported healthy volunteers, who are non-smokers and not on antiplatelet therapy (Supplemental Table I) that self-identified as either white or black. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation of whole blood for 12 minutes at 200 and 2000 g, respectively. The platelet concentration was measured using a Coulter Z1 particle counter (Beckman Coulter, Brea, CA). The platelet count of the PRP was adjusted to $3.0 \times 10^8$ platelets/mL by the addition of PPP. To acquire washed platelets, platelets were pelleted from PRP treated with acid citrate dextrose (2.5% sodium citrate tribasic, 1.5% citric acid, 2.0% D-glucose) and apyrase (0.02 U/ml) via centrifugation at 2000 g and resuspended in Tyrodes buffer (12 mM NaHCO₃, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH₂PO₄, 1 mM MgCl₂, 5 mM glucose, 10 mM HEPES) to a final concentration of $3.0 \times 10^8$ platelets/mL.

Platelet aggregation: Platelet aggregation was measured for eight minutes following stimulation under stirring conditions (1100 rpm) at 37°C using an aggregometer (Model 700D, Chronolog Corp, Havertown, PA). The agonist and concentration used are reported where appropriate. For studies using 2MeSAMP or aspirin, platelets were pretreated with the inhibitors for 20 or 40 minutes, respectively.

Calcium mobilization: Washed platelets were resuspended to a final concentration of $1.0 \times 10^6$ platelets/mL in Tyrodes buffer. Prior to PAR4 stimulation, platelets were incubated with Fluo-4 AM, a cell permeable Ca²⁺ sensitive dye, for ten minutes. The platelets were supplemented with 1 mM CaCl₂ then stimulated with PAR4-AP and mean fluorescence intensity (MFI) was measured in real-time on a flow cytometer for ten minutes to monitor the rise in free intracellular Ca²⁺. Data is reported as the fold change comparing maximum MFI to the baseline MFI measured prior to platelet stimulation.

Rap1 activation: Washed platelets were stimulated with PAR4-AP and lysed with 2X platelet lysis buffer (100mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% IGEPAL, 1% sodium deoxycholate, 0.05% sodium dodecyl sulfate (SDS), 2 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride, 2
mg/ml leupeptin, 2 mg/ml aprotinin) at indicated times. The active, GTP-bound form of Rap1 was selectively precipitated from the lysates of PAR4 stimulated platelets using a GST-tagged RalGDS Rap1-binding domain conjugated to glutathione agarose beads, as previously described.\textsuperscript{1} Total platelet lysate and Rap1 pull-down samples were run on a SDS-polyacrylamide gel electrophoresis (PAGE) gel and identified by Western blot with a Rap1 antibody. Band intensity was quantified with the Odyssey Infrared Imaging System (LI-COR Biosciences). The levels of active Rap1 were normalized to the amount of total Rap1 contained in each lysate.

**PKC activation:** Washed platelets were activated with PAR4-AP under stirring conditions and lysed at indicated times with 5X Laemmli buffer (300 mM Tris pH 6.8, 10% SDS, 50% Glycerol, 25% 2-mercaptoethanol, .05% bromophenol blue). Samples were separated on a SDS-PAGE gel and Western blot analysis was performed with PKC-substrate and Pleckstrin antibodies. The amount of PKC phosphorylation of Pleckstrin was normalized to the total amount of Pleckstrin in each sample. Data are presented as fold change relative to resting platelets.

**αIIbβ3 activation:** Washed platelets were incubated with a FITC-conjugated antibody specific for the active conformation of αIIbβ3, PAC-1. Platelets were stimulated by the addition of PAR4-AP and activation was stopped at the indicated times by the addition of 2% formaldehyde. The MFI of each PAR4 stimulated sample was measured by flow cytometry and αIIbβ3 activation was reported as fold change compared to the MFI of unstimulated platelets incubated with PAC-1.

**Western blot for protein expression:** Resting platelets (5.0 x 10\textsuperscript{8} platelets/mL) were lysed with 5X Laemmli buffer. The lysates were loaded on 10% SDS-PAGE gels and probed with antibodies specific for αIIb, Rap1, and GAPDH. The levels of αIIb, and Rap1 were normalized to the amount of GAPDH in each sample.

**Statistical analysis:** Statistical significance was calculated with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) using an either unpaired, one-tailed t-test or two-way ANOVA. Where appropriate, the data represent the mean ± S.E.M.

\textsuperscript{1} Holinstat M, Voss B, Bilodeau ML, McLaughlin JN, Cleator J, Hamm HE. Par4, but not par1, signals human platelet aggregation via ca2+ mobilization and synergistic p2y12 receptor activation. *The Journal of biological chemistry.* 2006;281:26665-26674
**SUPPLEMENTAL MATERIAL**

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**Supplemental Table I**: Comparison of the demographics of the white and black donors used for experiments in this study.
Supplemental Figure I: The aggregation of washed platelets from black or white donors was measured in response to increasing concentrations of thrombin (n >8) (A), PAR-1-AP (n >9) (B) or U46619 (n=8) (C). Data represents mean ± S.E.M (two-way ANOVA)
Supplemental Figure II: Representative tracing of washed platelets from black (black line) or white (dashed line) donors treated with aspirin and 2MeSAMP prior to stimulation with 100, 150 or 200 μM of PAR4-AP. Composite of this data is depicted in Figure 2c.