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Enhanced potency of prasugrel on protease-activated receptors following bivalirudin treatment for PCI as compared to clopidogrel

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Abstract

ACS patients undergoing percutaneous coronary intervention (PCI) when treated with bivalirudin and clopidogrel had increased frequency of early stent thrombosis. 24 patients referred for intervention with planned bivalirudin therapy, not previously treated with a P2Y₁₂ inhibitor and not receiving heparins or α IIb β 3 inhibitors were randomized to treatment with either clopidogrel (600 mg) or prasugrel (60 mg). Platelet aggregation (PA) was measured by light transmission aggregometry (LTA) of platelet-rich plasma in response to ADP, PAR1/PAR4 thrombin receptor agonists and collagen at baseline and at 1, 2, 4 and 16 h following the cessation of bivalirudin infusion. Prasugrel-mediated inhibition of PA was significantly greater than that of clopidogrel at all time points for ADP as well as PAR1. There was an unanticipated, significantly greater protection of PAR4-mediated platelet aggregation only detected with prasugrel and not observed with clopidogrel. We further examined the effect of the hyperreactive PAR4 Thr120 variant in the protease-activated receptor 4 (PAR4), single nucleotide polymorphism (SNP) rs773902 on aggregation protection. The PAR4 protective effect with prasugrel was lost in individuals carrying the PAR4 Thr120 variant, and not in Ala120 homozygote. PAR1, ADP and collagen inhibition was not significantly affected in the hyperreactive PAR4 Thr120 variant. We documented that the P2Y₁₂ ADP receptor-mediated regulation of the strength of the high-affinity conformation of α IIb β 3 as detected by PAC-1 ab, and in control of platelet adhesiveness through Rap1 GTPase protein activation. Importantly, the PAR4 Thr120 variant resulted in the increased rate and magnitude of Rap1 activation. Human platelet PAR4 mediated-activation of α IIb β 3 was phospholipase C beta (PLC β)-dependent and unlike mouse platelet PI3K-independent. These data

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identify a PAR4-dependent inhibitory mechanism for the prasugrel-mediated platelet inhibition, not seen with clopidogrel that could explain the reduction in stent thrombosis documented in clinical trials with prasugrel.

Keywords

P2Y₁₂; PAR4 SNP rs773902; Collagen; Platelets; Arterial thrombosis

1. Introduction

Percutaneous coronary intervention (PCI) leads to iatrogenic endothelial disruption and heightened platelet activation and aggregation. Treatment with heparin and glycoprotein (GP)IIb/IIIa (α IIB β 3) inhibitors during PCI effectively mitigates platelet aggregation but frequently increases bleeding [1–3]. In addition, there are several wellknown limitations of heparin including its variable anticoagulant effect due to nonlinear pharmacokinetics, inconsistent binding to blood proteins and ineffective inhibition of clot-bound thrombin as well as its propensity to lead to thrombocytopenia.

The direct thrombin inhibitor (DTI), bivalirudin, which binds with high affinity to the exosite I of thrombin, may be a safer alternative to other commonly used pharmacologic PCI adjuncts with the preponderance of evidence from randomized trials showing bivalirudin to be associated with less bleeding and thrombocytopenia, comparable ischemic events and improved net clinical outcomes compared with heparin-based regimens [4–6]. Other studies Results from our laboratory suggest that at least a part of the salutary effects of bivalirudin are due to a reduction of thrombin and to a lesser extent, collagen-mediated platelet activation [7,8].

Inhibition of the platelet P2Y₁₂ ADP receptor is the standard of care when added to aspirin in patients undergoing PCI. The more potent P2Y₁₂ ADP receptor antagonist prasugrel significantly reduced the composite endpoint of cardiovascular death, nonfatal MI, and nonfatal stroke in higher-risk ACS patients referred for PCI [7–9].

Treatment with bivalirudin alone, as compared with heparin plus α IIB β 3 inhibitors in ST-segment elevation myocardial infarction (STEMI), resulted in significantly reduced 30-day rates of major bleeding and net adverse clinical events [9], however bivalirudin treatment was associated with a higher acute stent thrombosis rate when compared to α IIB β 3 inhibitor-treated patients [9]. We have documented that the half-life of bivalirudin during PCI is 29.3 min [10]. The relatively short half-life of this DTI in concert with the relatively long time required to activate clopidogrel from a prodrug to its active metabolite [11], likely exposes patients to a vulnerable period when there is suboptimal platelet inhibition, which likely is the proximate cause of early stent thrombosis in bivalirudin-treated STEMI patients. Consequently, earlier acting, more potent thienopyridine therapy, i.e., prasugrel, when combined with bivalirudin treatment has the potential to reduce bleeding (compared with α IIB β 3 inhibitors) while preventing peri-procedural MI as well as providing protection from platelet-mediated stent thrombosis (compared with clopidogrel) during the vulnerable period following PCI.

The overwhelming majority of published data examining clinical outcomes or in-vivo pharmacodynamic and pharmacokinetic differences between clopidogrel and prasugrel have done so in PCI patients in whom bivalirudin was either not used or used very infrequently, i.e., in < 10% of studied patients [9,12,13]. However, at present in the United States, bivalirudin is a preeminent antithrombotic adjunctive therapy used during PCI [10]. Consequently, comparative data regarding the effect of prasugrel and clopidogrel on platelet function in bivalirudin-treated patients is of vital clinical importance.

This study aims to document the extent of inhibition of platelet aggregation following the discontinuation of bivalirudin therapy in PCI patients treated with prasugrel as compared with clopidogrel. To compare the effect of prasugrel and clopidogrel on platelet function in bivalirudin-treated patients, change from baseline in platelet aggregation in response to 5 and 20 μ M ADP, 5 and 20 μ M SFLLRN (PAR1), 160 and 300 μ M AYPGKF (PAR4) and 5 μ g/mL collagen concentrations were examined. ADP is an agonist for the dual ADP receptors, P2Y₁ and P2Y₁₂. Both, clopidogrel and prasugrel covalently and irreversibly bind to the P2Y₁₂ receptor and block its activation with distinct metabolic differences.

Thrombin, the most potent agonist of platelet activation enhanced factoring of PAR4 via PAR1, is, therefore, the most relevant agonist of PARs. The two thrombin-activated receptors on human platelets, the high-affinity PAR1 and the low-affinity PAR4 result in activation of the fibrinogen receptor α IIb β 3 that leads to platelet aggregation. We have previously shown that PAR1 and PAR4 form a stable heterodimer that enables thrombin to act as a bivalent functional agonist that results in acceleration of thrombin cleavage [7,14]. PAR4 is cleaved more slowly than PAR1 mainly because it lacks a functional Hir sequence [15]. The cleaved PAR1 exodomain retains the Hir motif and binds to exosite I [16], suggesting that thrombin may remain tethered to the surface of human platelets via its association with the cleaved PAR1 receptor. Because of its therapeutic importance as an antiplatelet target, the central role of the P2Y₁₂ receptor [17] in platelet activation and its contribution to the switch from a low-affinity state to a high-affinity state of α IIb β 3 that binds fibrinogen is under investigation. Our group and others have previously demonstrated inhibition of P2Y₁₂ affected PAR1 signaling and contributed to 'irreversible' platelet aggregation [18,19]. Previous work on mouse platelets identifies that PAR4 and the P2Y₁₂ ADP receptor signal synergistically in platelet activation [20,21]. Therefore thrombin-mediated PAR1-PAR4 activation and other G-protein coupled receptors such as P2Y₁₂ have also been shown to form dimers with PAR4 and present a precisely orchestrated set of events in platelet activation and signaling that controls the precise balance between thrombosis and hemostasis.

PAR4 variant [22,23] with a single nucleotide polymorphism that results in transmembrane 2 residue amino acid 120 to code for a Threonine (Thr) or Alanine (Ala), rs773902 allele (a single nucleotide dimorphism G/A) of the PAR4 gene (*F2RL3*) was associated with different platelet reactivity. PAR4 variant Thr120 had increased PAR4 reactivity and higher platelet aggregation response. We postulated that the variability in PAR4 function due to genetic variants (Thr vs. Ala120) could affect the antiplatelet effects of P2Y₁₂ drugs.

Sustained integrin α IIb β 3 activation on a platelet surface is tightly regulated in the formation of a stable hemostatic plug and in a pathologic thrombus formation through the small GTPase RAP1 [24]. P2Y₁₂ is a key regulator of sustained RAP1 activation critical for the modulation of platelet adhesiveness. The RAP1 activator CalDAG-GEFI and inhibitor RASA3 are critical regulators that ensure proper activation of α IIb β 3. Human PAR4 signaling pathway and the role of Gi-coupled receptors and its contribution to Rap1 activation that modulates the strength and duration of irreversible α IIb β 3 activation are still not well understood, especially in the context of the PAR4 genetic variants. Here we provide evidence for the critical role of P2Y₁₂ in PAR4-mediated platelet aggregation uncovered with the more potent P2Y₁₂ drug prasugrel. The PAR4 protective effect with prasugrel was lost in individuals carrying the PAR4 Thr120 variant, and not in Ala120 homozygote. These findings are consistent with the recent report on effects of genetic variation in PAR4 on the ACT from the TRACER trial that detected a lower rate of GUSTO moderate/severe bleeding in patients with the PAR4 Thr120 [25].

2. Methods

2.1. Patient population and recruitment

This study examined the antiplatelet effects in PCI patients randomized to treatment with clopidogrel vs. prasugrel during the vulnerable period following the discontinuation of bivalirudin treatment, an approved adjunctive agent for PCI employing its commonly-used dose in the approved patient population for this drug. Patients referred for PCI who had not received bivalirudin and thienopyridine therapy and had not been exposed to P2Y₁₂ inhibitors within 2 weeks were enrolled in the Tufts Medical Center Adult Cardiac Catheterization Laboratory.

Inclusion criteria included men and non-pregnant women, age 18 years or older referred for possible PCI who were eligible for bivalirudin therapy. All patients provided written informed consent before the initiation of the study. The study protocol was approved by the Tufts Medical Center Institutional Review Board. Heparin was not administered for at least 4.5 h before or during the intervention. Patients could not be currently receiving α IIb β 3 inhibitors and have no contraindications to bivalirudin. Exclusion criteria included age \geq 75 years, weight $<$ 60 kg, pregnancy, serum creatinine $>$ 2.0, platelet count $<$ 50,000/ μ L, known hypersensitivity to bivalirudin, clopidogrel, prasugrel or aspirin, severe systemic hypertension, defined as SBP $>$ 180 mmHg or DBP $>$ 110 mmHg, symptoms or findings suggestive of aortic dissection, acute pericarditis, cardiogenic shock, active internal bleeding or a history of ICH, stroke, TIA, AV malformation or aneurysm, any major surgical procedure or severe physical trauma and participation in other clinical research studies within 30 days of enrollment.

Eligible patients received bivalirudin administered intravenously as a 0.75 mg/kg bolus followed by continuous infusion of 1.75 mg/kg/h during the procedure. All patients were chronic aspirin users and received it before the intervention. Oral clopidogrel (600 mg) or prasugrel (60 mg) was administered in an open-label fashion at the completion of PCI and were discharged with a prescription for either 75 mg clopidogrel or prasugrel 10 mg daily, per usual clinical practice.

2.2. Platelet aggregation studies

Venous blood was collected from 24 patients with an 18-gauge needle and 20 cc syringe pre-filled with 200 μ M PPACK and were transferred to 15 mL polypropylene tubes. Platelet-rich plasma (PRP) was extracted after centrifuging the blood at 800 rpm for 20 min at 30 °C. Platelet-poor plasma (PPP) was, used as a baseline for light transmission and was obtained after PRP removal and centrifuging the samples at 3000 rpm for 10 min. Platelet aggregation was measured in PRP, in a blinded fashion after induction with various agonists: ADP 5 and 20 μ M, SFLLRN 5 and 20 μ M, AYPGKF 160 μ M and 300 μ M, collagen 5 μ g/mL in PRP. Aggregation was measured blinded to treatment assignment using a Chronolog 560VS/490 to 2D aggregometer. Reactions were conducted in final volumes of 250 μ L at 37 °C, stirring at 900 rpm.

2.3. Prothrombinase and TAT assays

Quantification of thrombin-antithrombin III complexes (TAT) in plasma from patients was obtained at baseline, 1 h, 2 h, 4 h, and 16–24 h after cessation of bivalirudin infusion. Briefly, the patient plasma was prepared by centrifuging the test tube at 3000 rpm at 37 °C and storing it at –80 °C until analysis. Assays were performed by ELISA (Affinity Biologicals, Cayman Chemicals, respectively).

2.4. Measurement of PAC-1 binding to evaluate α IIb β 3 activation through PAR4-P2Y₁₂

Activation of α IIb β 3 was measured in whole blood by flow cytometry. Whole blood collected from healthy donors, PRP was prepared, and GPRP at a final concentration of 1 mM was added to prevent fibrin polymerization before the addition of agonists. PRP was incubated for 30 min with 1 U/mL apyrase, 10 μ M AZD1283 [26] (BioVision), 10 μ M U73122, 30 μ M LY294002 and 30 μ M GF109203X then incubated for an additional 15 min with 160 μ M AYPGKF, PAR4 peptide agonist at 37 °C. The samples were labeled with PAC1-FITC (BD Biosciences) ab in the dark at room temperature for 30 min. Paraformaldehyde 1% was used to fix the platelets. The analysis was performed on a BD FACS Canto II Flow Cytometry System.

2.5. Rap1 activation assay

Gel-purified platelets cells were stimulated with 160 μ M AYPGKF for 5 min, 15 min, 30 min, 1 h at 37 °C and 0 time (vehicle control). Samples were pretreated platelets with 30 μ M AZD1283, +160 μ M AYPGKF for 5 min, 15 min, 30 min, 1 h at 37 °C and 0 time (vehicle control). Prepared platelet lysates using extraction lysate buffer (25 mM tris(hydroxymethyl)aminomethane (Tris)-HCl at pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT (dithiothreitol), 5% Glycerol, 1 g/mL aprotinin, 1 g/mL leupeptin, and 1 mM phenyl methylsulphonyl fluoride (PMSF)) on ice for 10 min, cells were centrifuged at 14,000 *g* for 10 min at 4 °C. Aliquots of the lysate were used to detect total Rap1 levels. The remaining supernatants were incubated with 50 μ L of Ral GDS-RBD agarose slurry of (EMD Millipore, MA) for 60 min at 4 °C. Proteins were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Rap1 levels were detected

with rabbit polyclonal antibodies (EMD Millipore, MA) followed by horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibodies (Zymed, South San Francisco, CA).

2.6. Genotyping PAR4 rs773902

Genotyping of PAR4 rs773902 A allele and/or G was carried out by performing polymerase chain reaction analysis and sequencing of the amplified fragment that scans the PAR4 rs773902 SNP.

2.7. Western blot analysis of patient samples for P2Y₁₂ expression

P2Y₁₂ ADP receptor expression was measured by western blot from platelet samples at baseline and 1 h time points. PRP patient samples at baseline and 1 h were centrifuged at 3000 rpm for 10 min, and the supernatant was removed. The remaining pellet was stored at –80 °C. These samples were then thawed and lysed in lysate buffer (100 mM NaCl, 25 mM Hepes pH 7.2, 1% Nonidet P-40, 1 mM PMSF) with Halt-Protease Inhibitor mixture (Thermo Scientific) and protein was quantified using a Bradford assay. The samples were then run on 12% SDS-PAGE. Rabbit polyclonal P2Y₁₂-Ab was directed against the P2Y₁₂ receptor C-terminal region residues 13–26, was generated as previously described [27]. Membranes were incubated with anti-rabbit Ig-horseradish peroxidase antibody (Dako A/S, Glostrup, Denmark 1:1000 dilution) for 1 h at room temperature. Membrane proteins were detected by enhanced chemiluminescence (Amersham Pharmacia) and exposed on Hyperfilm (Amersham Pharmacia). Protein from western blots was quantified using densitometry with ImageJ and actin was used as the correction factor for loading.

3. Statistical analysis

Based on previous studies from our group [10,28] 24 total patients will provide 95% power to detect statistically significant differences between baseline and prasugrel and clopidogrel post-bivalirudin infusion for inhibition of aggregation to various agonists including ADP, SFLLRN, AYPGKF and collagen agonists with a standard deviation (SD) of 10–24%. From Li [29] et al., and Michelson [13] et al., a mean inhibition of 30% with SD 15% in ADP aggregation at timepoint 1–4h after prasugrel 60 mg or clopidogrel 600 mg would yield a power of 99.8%. From prior work in our lab [10], a 30% mean inhibition in collagen inhibition at time points 1–4 h after prasugrel 60 mg or clopidogrel 600 mg would yield a power of 92.4%. A 15% mean inhibition in SFLLRN would yield a power of 95.7%. Finally, a 20% mean inhibition in AYPGKF would yield a power of 90.4%. One-way ANOVA repeated measure test was used for statistical analysis of aggregometry results. All other statistical analyses were performed using two-tailed student's *t*-test. Statistical significance was assumed to occur at $p < 0.05$.

4. Data analysis

Each patient had paired samples representing their baseline as well as an on-drug sample. The magnitude of platelet inhibition for each studied agonist was performed utilizing mean maximal change from baseline in LTA. The paired samples were analyzed using a two-tailed student's *t*-test. Statistical significance was assumed to occur when $p < 0.05$. Mechanistic

studies are explicitly documenting the effect of prasugrel versus clopidogrel on ADP, PAR1 and PAR4 thrombin receptors, and collagen-receptors were performed. Statistical significance was assumed to occur when $p < 0.05$. One-way ANOVA repeated measure test was used for statistical analysis of aggregometry results.

5. Results

24 patients referred for PCI that were eligible for bivalirudin therapy were randomly assigned in a 1:1 ratio to treatment with either clopidogrel (600 mg loading dose) or prasugrel (60 mg loading dose). Patient baseline characteristics are listed in Table 1. There were no major or minor bleeding events according to Thrombolysis In Myocardial Infarction (TIMI) criteria [2] nor any other complications. PA was measured by light transmission aggregometry (LTA) of platelet-rich plasma in response to ADP, PAR1/PAR4 thrombin receptor agonists and collagen at baseline and at 1, 2, 4 and 16 h following the cessation of bivalirudin infusion as schematically indicated in Fig. 1.

5.1. Prasugrel uncovers PAR4-P2Y₁₂ synergism in platelet aggregation

P2Y₁₂ and the low-affinity thrombin receptor, PAR4 form a heterodimer, the physical association between these two receptors has been previously reported [30]. We have previously shown that individuals with Hermansky-Pudlak Syndrome that lack platelet dense granules and have no ADP-autocrine response have mild bleeding and unlike PAR1 still have irreversible platelet aggregation [18]. Consistent with this report, saturating concentrations of the PAR4 agonist AYPGKF (300 μ M) did not reveal a significant role for P2Y₁₂ in PAR4 activation (data not shown). However, prasugrel was more potent at inhibiting platelet aggregation in the presence of the PAR4 agonist, AYPGKF at a lower concentration of 160 μ M up to 53% as compared to 13% inhibition with clopidogrel ($p < 0.0001$)(Fig. 2B, Table 2). Therefore, prasugrel uncovers PAR4-P2Y₁₂ synergy when sub-saturating concentrations of a PAR4 agonist (160 μ M) are used. Inhibitory effect of prasugrel may be an important finding and warrants further investigation for the variability in antiplatelet therapy protection with prasugrel and its contribution to PAR4 activation.

5.2. Prasugrel inhibition is superior to clopidogrel in suppressing PAR1-induced aggregation in platelets from patients undergoing PCI

Next, we determined the effects of clopidogrel and prasugrel on the activity of the high-affinity thrombin receptor, PAR1 using SFLLRN, the agonist of PAR1, and assessed aggregation in PRP from patients undergoing PCI. It has been previously reported that upon PAR1 activation, the dense granule-secreted ADP acts as an autocrine signal to activate the G_i-coupled P2Y₁₂ receptor and 'lock' the integrin α IIb β 3 receptor in irreversible aggregation [18]. Consistent with these reports as shown in Fig. 3A, there is almost complete normalization of activation measured at the early time point (~20% inhibition with prasugrel and ~5% with clopidogrel) observed with 5 μ M SFLLRN. There is up to 75% final inhibition ($p < 0.0001$) of PAR1 activation with prasugrel which is significantly more potent than that observed with clopidogrel (17%) (Fig. 3B, Table 2). When saturating levels of SFLLRN (20 μ M) were used as the agonist, the inhibition of platelet aggregation was reduced to 30% with prasugrel, and negligible inhibition was detected with clopidogrel <

5% (data not shown). Therefore, superior P2Y₁₂ inhibition mediated by prasugrel resulted in more reversible (dissociable) platelet aggregation to the potent PAR1 agonist, SFLLRN.

5.3. Prasugrel inhibition is superior to clopidogrel in suppressing ADP-induced aggregation in platelets from patients undergoing PCI

Bivalirudin binds with high affinity to the exosite I of thrombin and is used as an adjunctive antithrombotic therapy in patients undergoing PCI [10]. Termination of the bivalirudin infusion results in rapid clearance of the drug with a half-life of 29.3 min [10]. Two hours after discontinuation of the bivalirudin infusion, residual plasma drug levels (0.2 μM) were only 11% of the steady-state levels. Therefore following discontinuation of bivalirudin therapy, the postulated vulnerable window for acute thrombotic events following PCI is between 1 and 4 h. To compare the effect of prasugrel and clopidogrel on platelet function in bivalirudin-treated patients, change from baseline in platelet aggregation in response to 5 and 20 μM ADP concentrations were examined first. Both clopidogrel and prasugrel covalently and irreversibly bind to the P2Y₁₂ receptor and block its activation with distinct metabolic differences. Clopidogrel is metabolized by a two-step mechanism dependent on CYP enzymes, whereas the faster-acting prasugrel uses a single oxidative step resulting in a more rapid onset of activity [11]. Consistent with previous publications, prasugrel was associated with increased inhibition of maximum and final platelet aggregation to the P2Y₁₂ agonist ADP at both 5 and 20 μM concentrations (Fig. 4, Table 2) ($p < 0.0001$). The difference in inhibition was seen at every time point examined compared to baseline. The differences were most marked during the early 1–2 h period where maximum inhibition of 94–98% was already achieved with prasugrel as compared to clopidogrel where the maximum inhibition of P2Y₁₂-mediated platelet aggregation was 85% at 16 h post-loading dose in response to either 5 μM or saturating ADP levels (20 μM) ($p < 0.0001$). These data demonstrate that more rapidly-acting prasugrel gives a superior maximum and sustained protection within 1–2 h after a loading dose and its protection is persistent over the 16 h measured time period.

We examined the effect of clopidogrel and prasugrel on protein expression of P2Y₁₂, the receptor on human platelets 1 h after administration of both thienopyridines when compared with the basal expression before treatment. There was a non-significant increase in P2Y₁₂ protein expression post administration of both clopidogrel and prasugrel (Suppl. Fig. 1).

5.4. Prasugrel is protective against collagen-mediated platelet activation as compared to clopidogrel during the early vulnerable period

P2Y₁₂ has a prominent role in collagen-induced human platelet aggregation. Prasugrel was fast-acting and inhibited by 56% ($p = 0.06$) within the first hour of the addition of collagen (5 μg/mL), that persisted during the vulnerable period (Fig. 5, Table 2), and its inhibition was sustained throughout the vulnerable 1–2 h period. In contrast, arguable clopidogrel was less protective (Table 2) within the 1 to 2 h post-termination of bivalirudin time-frame as compared to prasugrel. At later time points from 4 h to 16 h, prasugrel inhibition was not significantly different from clopidogrel.

Antithrombin III irreversibly binds to active thrombin to rapidly form TAT complexes which are an indirect estimate of systemic thrombin levels. Because decreased platelet activity observed with various agonists was superior with prasugrel, we measured systemic thrombin levels following administration of the loading dose of the two drugs in the patients undergoing PCI. The baseline mean systemic levels of TAT were similar in both groups of PCI patients randomized to treatment with clopidogrel vs. prasugrel (258 ± 39 and 251 ± 27 pmol/L, respectively). We compared plasma TAT levels at 1, 2, 4 and 16 h. As shown in Suppl Fig. 2 the PCI patients had the largest drop in mean systemic TAT levels at 16 h (151 ± 10 pmol/L, Table 2) following treatment with clopidogrel. There was a trend of protection at 1, and 4 h that was not significant with the prasugrel treatment at 16 h time point. However, the small sample size is an important limitation in examining TAT levels.

5.5. Effect of PAR4 Thr120 vs Ala120 genetic variation in platelet aggregation and contribution of P2Y₁₂

The rs773902 SNP was genotyped in 23 patients and showed that 27% were G/G or A/G genotype that codes for the hyperactive Thr120 PAR4 isoform in prasugrel treatment group whereas clopidogrel group had 17%. There was a striking reversal in inhibition detected by prasugrel treatment in the individuals with the hyperactive Thr120 PAR4 (homozygous and heterozygous) as compared to the individuals with homozygous Ala120 PAR4 (Fig. 6A, B) detected with PAR4 ligand AYPGKF activation. Interestingly, the reversal in platelet aggregation inhibition was most prominent for the PAR4 inhibitory effect of prasugrel and with some increased variability for PAR1 inhibitory effect in the hyperactive Thr120 PAR4 (Fig. 6C, B). There was a minor effect on collagen-mediated aggregation in clopidogrel treatment only, whereas prasugrel treatment did not seem affected (Fig. 6E, F).

5.6. PAR4-mediated α IIB β 3 activation and P2Y₁₂ contribution to PAC-1 and Rap1 activation and P-selectin expression

To further investigate the contribution of the Gi-coupled P2Y₁₂ receptor to activation of integrin α IIB β 3, conformational changes were examined using flow cytometry studies with anti-PAC1 Ab that recognizes the activated form of α IIB β 3. The input of the P2Y₁₂ receptor to the contribution of PAR4 mediated fibrinogen receptor activation was examined using a potent direct P2Y₁₂ drug inhibitor AZD1283 [31]. As shown in Fig. 7A, there was a 50% reduction in intensity of PAC-1 binding in the presence of direct drug inhibitor AZD1283. In contrast, there were less marked differences in the contribution of P2Y₁₂ to P-selectin expression (Fig. 7C).

Next, we determined whether a downstream signaling mechanism of α IIB β 3 activation by PAR4 was PLC β , PKC and/or PI3K dependent. PAC-1 binding was inhibited with U73122 (PLC β inhibitor), but not affected with LY294002 (PI3K inhibitor), nor GF109203X (PKC inhibitor) therefore identifying the phospholipase C beta (PLC β)-dependent signaling mechanism as the primary mechanism for α IIB β 3 activation by PAR4 (Fig. 7B). Both, PLC β and PKC PAR4-mediated activation were important for P-selectin expression (Fig. 7D).

In order to determine the effect of SNP rs773902 on PAR4 mediated activation of Rap1, a direct activator and critical regulator of platelet adhesiveness and formation of a stable

hemostatic plug is P2Y₁₂ dependent; we examined the effect of PAR4 activating peptide AYPGKF on Rap1 platelet activation in the presence of direct P2Y₁₂ drug inhibitor AZD1283 over time. PAR4 activating peptide AYPGKF results in rapid and sustained Rap1-GTP activation as examined over 60 min (Fig. 7E) with robust activation and duration detected in the hyperactive homozygous and heterozygous Thr120 PAR4. The duration of Rap1 activation in the homozygous Ala120 PAR4 was rapid with fast 'on' and 'off' state. In the presence of the P2Y₁₂ inhibitor AZD1283, there is complete inhibition of AYPGKF activation of Rap1 only in the homozygous Ala120 PAR4, whereas there is partial inhibition of the hyperactive Thr120 PAR4 detected. Together both PAC-1 and Rap1 data strongly identify P2Y₁₂ as an essential regulator of PAR4-mediated platelet activation especially critical in the homozygous Ala120 PAR4 and identify the effect of SNP rs7773902 on PAR4 as critical in the formation of a stable hemostatic plug. These studies warrant further investigation in individual differences and the effect of the PAR4 variants on the development of antiplatelet therapies.

6. Discussion

In this study, we documented a significantly higher inhibition of platelet aggregation by prasugrel as compared with clopidogrel – a difference that was most evident early following the discontinuation of bivalirudin therapy. As has been previously reported [11], a more rapid therapeutic thienopyridine effect can be expected to mitigate the potential for acute stent thrombosis. The more rapid appearance of prasugrel in the plasma compared to clopidogrel led to earlier and more potent inhibition of thrombin-mediated platelet activation. The data imply that prasugrel, when compared to clopidogrel has an important additive effect on the inhibition of platelet aggregation via inhibition of the PAR4 receptor following the termination of bivalirudin treatment. However, upon further examination, the effect of the hyperreactive PAR4 Thr120 variant in the protease-activated receptor 4 (PAR4), single nucleotide polymorphism (SNP) rs773902 on aggregation protection the PAR4 protective effect with prasugrel was lost in individuals carrying the PAR4 Thr120 variant, and not in Ala120 homozygote. PAR1, ADP and collagen inhibition was not significantly affected in the hyperreactive PAR4 Thr120 variant.

Acute stent thrombosis is an important clinical complication of PCI. It has been noted disproportionately during PCI for STEMI where the widely-used direct thrombin inhibitor, bivalirudin is used [32,33]. Although this complication appears to be prevented when the bivalirudin infusion is extended following the PCI at the procedural dose [34], it does appear that there exists a window of vulnerability as relates to a lack of platelet inhibition when PCI patients are concurrently treated with bivalirudin and clopidogrel. Lack of platelet inhibition is especially germane given the documented short half-life of bivalirudin [10]. The thienopyridine prasugrel has greater potency in inhibition of platelet aggregation when compared to clopidogrel [35,36]. Consistent with these previous reports prasugrel compared to clopidogrel therapy causes greater and earlier inhibition of platelet aggregation in response to both P2Y₁₂ and thrombin receptor agonists. This is the first report of significant inhibition of both PAR4 thrombin receptors with prasugrel as compared to clopidogrel in individuals that carry the Ala120 PAR4 phenotype.

The two thrombin-activated receptors on human platelets, the high-affinity PAR1 and the low-affinity PAR4 result in activation of fibrinogen receptor α IIb β 3 that leads to platelet aggregation. Because of its therapeutic importance as an antiplatelet target, the central role of the P2Y₁₂ receptor [17] in platelet activation and its contribution to the switch from a low-affinity state to a high-affinity state of α IIb β 3 that binds fibrinogen is under investigation. A proposed mechanism for the prasugrel-mediated reduction in stent thrombosis through inhibition of dual thrombin receptors is schematically shown in Fig. 8. Initial platelet generation of thrombin and binding to the high-affinity thrombin receptor PAR1 through interaction with the PAR1 hirudin (Hir)-like sequence results in PAR1 activation and release of dense granule ADP. ADP-mediated activation of P2Y₁₂ further activates platelets in synergy with PAR1 that results in a shift from reversible to irreversible platelet aggregation by activation of inside-out-signaling of the fibrinogen receptor, α IIb β 3 integrin. Inside-out signaling induces a change in conformation of the α IIb β 3 integrin leading to high fibrin binding and recruitment of additional platelets linking them together to form an irreversible platelet aggregate through activation of PAR4 mediated Rap1-GTP and its regulators CalDAG-GEF1 and RASA3. Human platelet PAR4 mediated activation of α IIb β 3 was phospholipase C beta (PLC β)-dependent with some potential role for PKC. Therefore, reinforcement of the platelet aggregate occurs as a result of thrombin activation of the low-affinity thrombin receptor, PAR4. Fast onset of inhibition of ADP signaling with the enhanced P2Y₁₂ inhibitor prasugrel uncovered a new role for P2Y₁₂/PAR4 in forming a more stable, growing platelet aggregates which contribute to both enhanced hemostasis and thrombosis. Both clopidogrel and prasugrel efficiently block PAR1-mediated platelet aggregation. In contrast, prasugrel more completely inhibits the signaling of the second thrombin receptor, PAR4, which may provide the basis for a more effective reduction in stent thrombosis. The dominant effect of Thr120 PAR4 variant showed incomplete inhibition of P2Y₁₂/PAR4, which may suggest another unknown signaling mechanism of Rap1/ α IIb β 3 integrin activation in the PAR4 hyperactive individuals.

The P2Y₁₂ receptor has a vital role in collagen-mediated platelet aggregation. Besides, we have previously demonstrated significant inhibition of collagen-mediated platelet aggregation by bivalirudin [10]. During the vulnerable period for patients, early following the termination of bivalirudin infusion, prasugrel was significantly more effective than clopidogrel in inhibiting collagen-mediated platelet aggregation. Thus, the more potent antiplatelet effect of prasugrel as compared to clopidogrel is likely multifactorial.

Overall, based on all of our aggregation data, prasugrel is a more potent platelet inhibitor than clopidogrel. P2Y₁₂ has a prominent role in collagen-induced human platelet aggregation. Prasugrel was faster acting as compared to clopidogrel and significantly inhibited collagen-mediated platelet aggregation within the first hour, therefore, protecting during the vulnerable period of stent thrombosis due to a lack of platelet inhibition immediately post-PCI prior to the onset of clopidogrel effect as plasma levels of bivalirudin quickly decline. In contrast, clopidogrel at later time points from 4 h to 16 h, platelet inhibition was not significantly different from prasugrel.

A limitation of this study is that patients randomized to each thienopyridine were treated in an open-label fashion. As light transmission aggregometry and other assays were done

blinded to of treatment assignment, it is unlikely that the open-label design biased our conclusions.

In this clinical study (24 patients), we documented a significantly higher inhibition of platelet aggregation by prasugrel as compared with clopidogrel – a difference that was most evident early following the discontinuation of bivalirudin therapy. These data imply that prasugrel when compared to clopidogrel, has a critical additive effect on the inhibition of platelet aggregation via inhibition of the PAR4 receptor. Upon further examination, the effect of the hyperreactive PAR4 Thr120 variant, single nucleotide polymorphism (SNP) rs773902 on aggregation, the PAR4 protective effect with prasugrel was lost in individuals carrying the PAR4 Thr120 variant, but not in the Ala120 homozygote. PAR4 activating peptide AYPGKF results in rapid and prolonged Rap1-GTP activation in the hyperactive homozygous and heterozygous Thr120 PAR4. The duration of Rap1 activation in the homozygous Ala120 PAR4 was rapid and transient. In the presence of the P2Y₁₂ inhibitor AZD1283, there is complete inhibition of AYPGKF activation of Rap1 in the homozygous Ala120 PAR4, whereas only partial inhibition of the hyperactive Thr120 PAR4 was observed, that may suggest of an additional P2Y₁₂-independent signaling mechanism. Together, these data strongly identify the effect of SNP rs773902 on P2Y₁₂ contribution as critical in the formation of a stable hemostatic plug.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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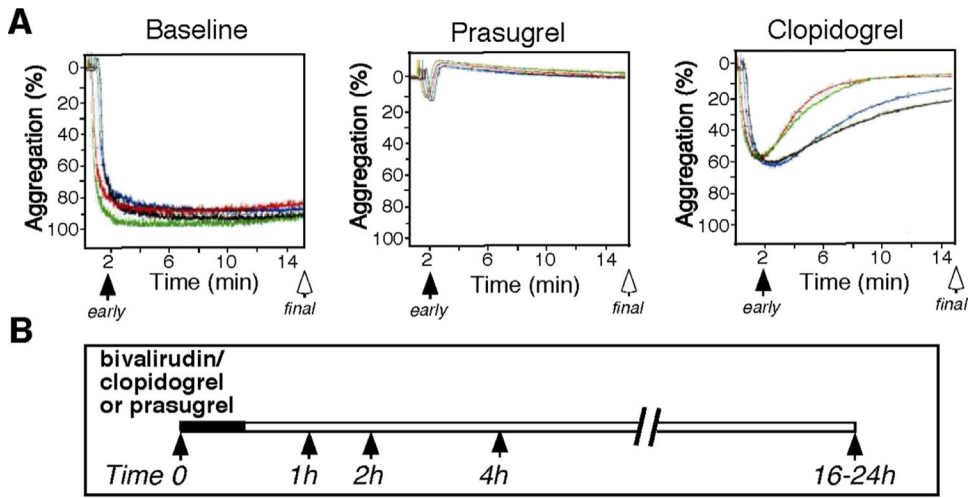


Fig. 1. Schematic of the blood samples collected during the study (time 0, 1, 2, 4 and 16–24 h) post bivalirudin administration. A. Inhibition of platelet aggregation was compared at early 2 min and final 15 min time point for platelets stimulated with agonist during the study (B) (time 0, 1, 2, 4 and 16–24 h). Patients were pretreated with Clopidogrel (n = 12) or Prasugrel (n = 12). PRP was prepared from patients at baseline (time 0) and at 1, 2, 4 and 16 h after discontinuation of bivalirudin. Platelet aggregometry was performed at 37 °C.

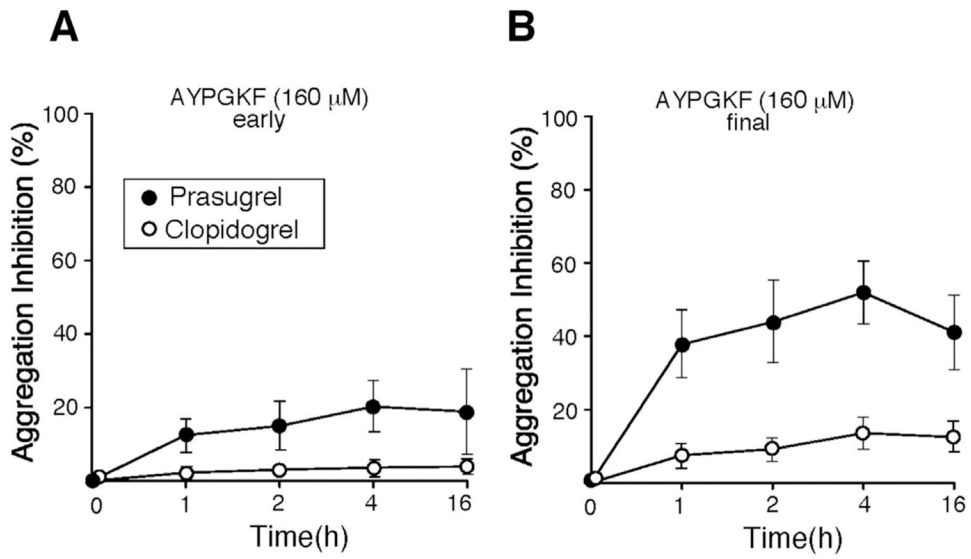


Fig. 2. Prasugrel uncovers the role of P2Y12 in PAR4-mediated platelet activation. Inhibition of platelet aggregation was compared at early 2 min (A) and final 15 min (B) time point for platelets stimulated with 160 μM AYPGKF. Data are reported as mean ± SE (n = 12). Prasugrel (●), Clopidogrel (○).

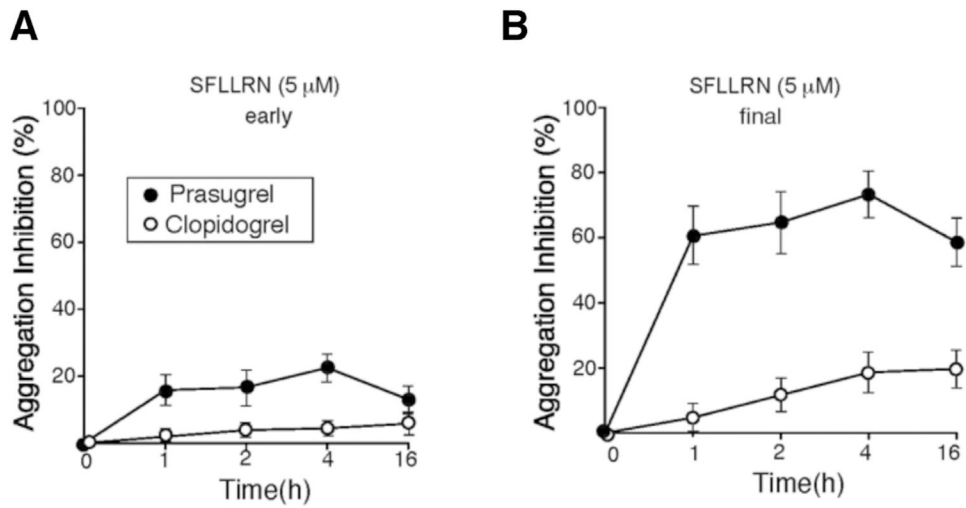


Fig. 3. Prasugrel is more potent inhibitor than Clopidogrel of platelet PAR1-mediated platelet aggregation. Inhibition of platelet aggregation was compared at early 2 min (A) and final 15 min (B) time point for platelets stimulated with 5 μ M SFLLRN. Data are reported as mean \pm SE (n = 12). Prasugrel (●), Clopidogrel (○).

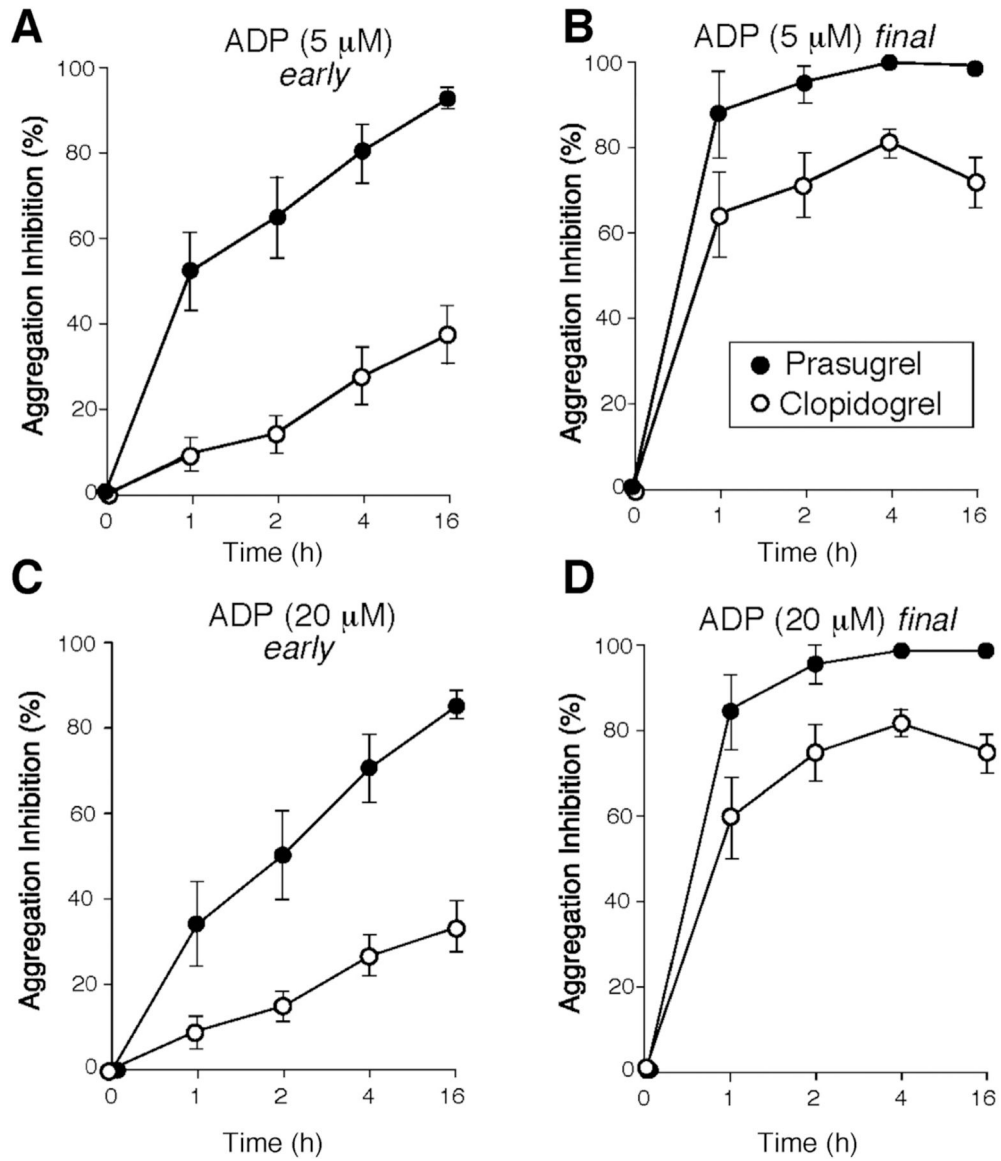


Fig. 4. Inhibition of P2Y₁₂-dependent platelet aggregation in after addition of ADP. Inhibition of platelet aggregation was compared at early 2 min (A, C) and final 15 min (B, D) time point for platelets stimulated with 5 and 20 μ M ADP (as indicated). Data are reported as mean \pm SE (n = 12). Prasugrel (●), Clopidogrel (○).

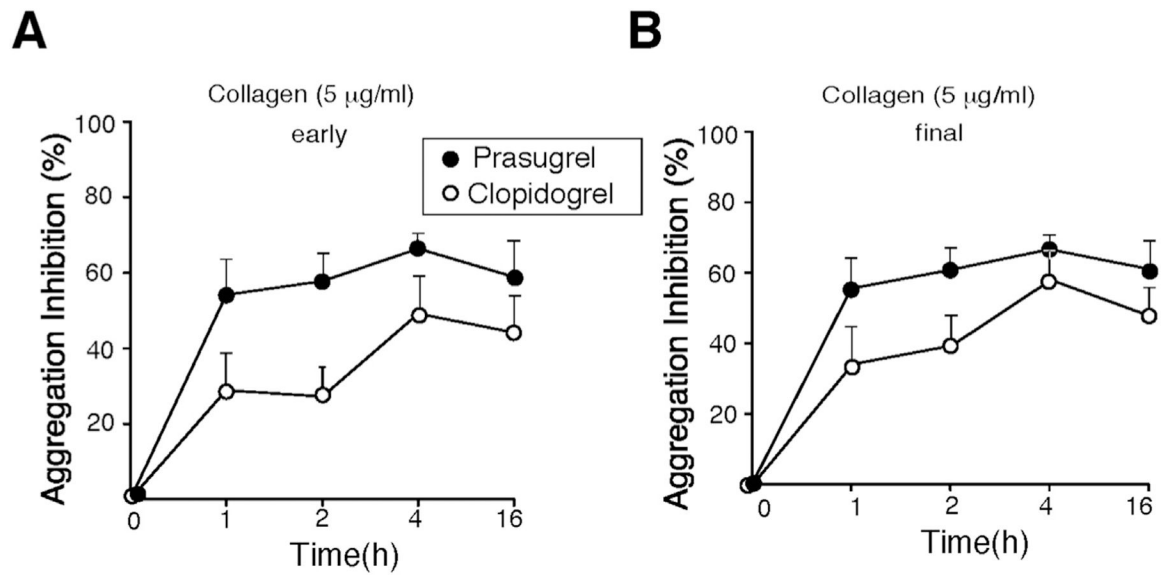


Fig. 5.

Prasugrel is more protective during an early vulnerable period as compared to Clopidogrel in Collagen mediated platelet activation. Inhibition of platelet aggregation was compared at early 2 min (A) and final 15 min (B) time point for platelets stimulated with 5 µg/ mL of collagen. Data are reported as mean ± SE (n = 12). Prasugrel (●), Clopidogrel (○).

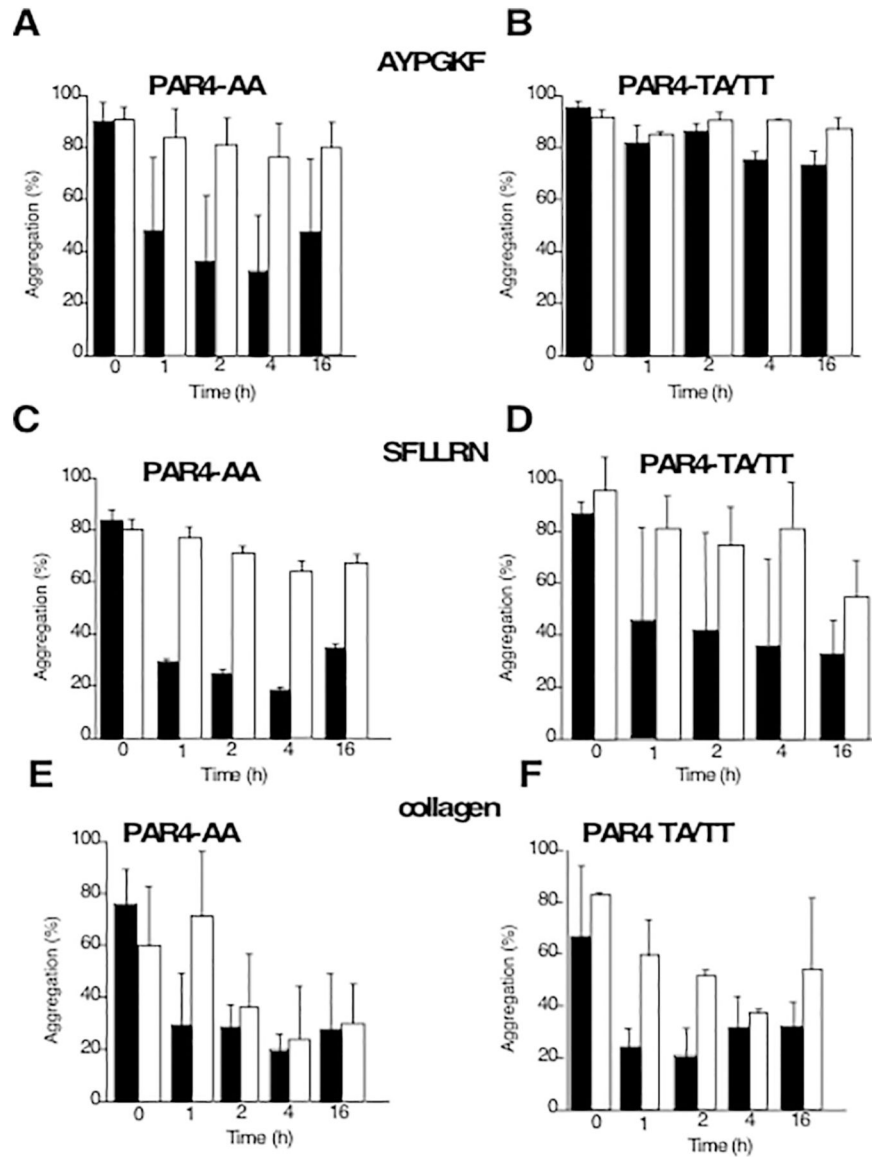


Fig. 6. Effect of PAR4 Thr120 vs Ala120 Genetic Variation in Platelet Aggregation and Contribution of P2Y₁₂. Platelet aggregation was compared at final 15 min time point for platelets stimulated with 160 μ M AYPGKF (A, B), 5 μ M SFLLRN (C,D) and 5 μ g/mL of collagen (E,F). Data are reported as mean \pm SD (n = 8–9 PAR4-AA, n = 2–3 PAR4-TA/TT). PAR4-AA: PAR4 genotype homozygous Ala120; PAR4-TA/TT: PAR4 genotype for the hyperactive Thr120 (TA-homozygote and TT heterozygote). Prasugrel (black), Clopidogrel (white).

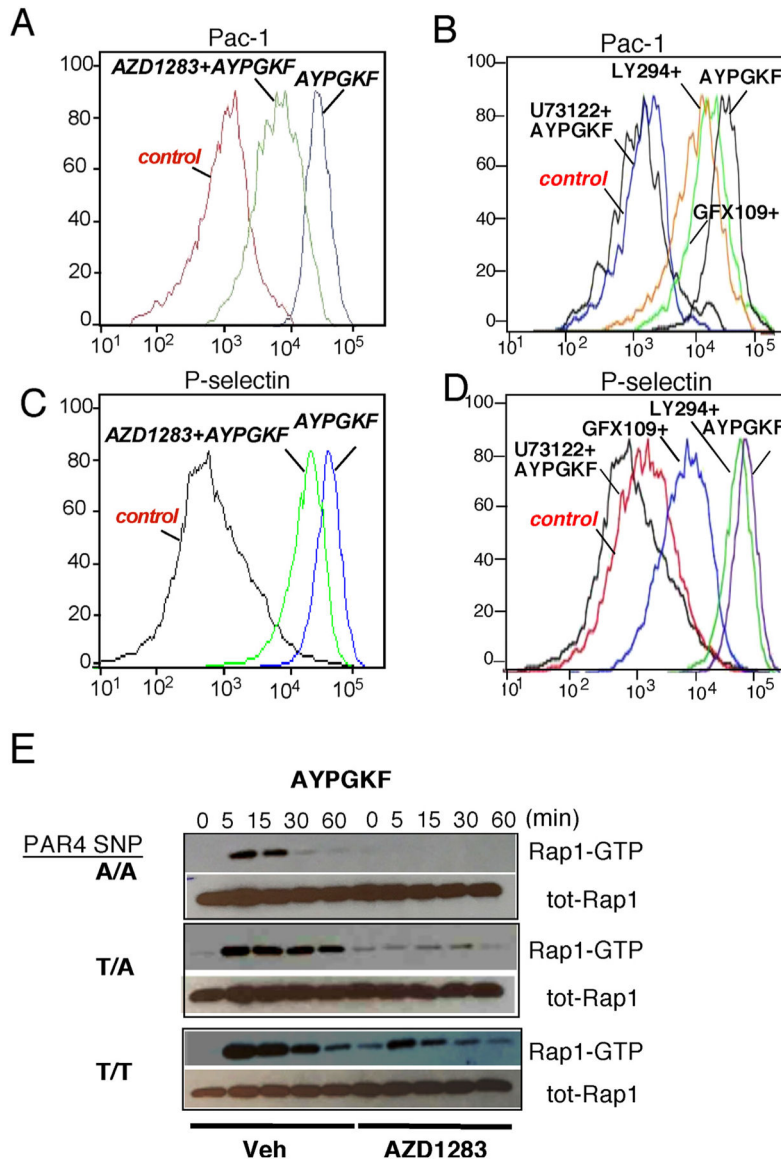


Fig. 7. PAR4/P2Y₁₂ mediated α IIb β 3 and Rap1 activation and P-selectin expression. PAC-1 binding: platelets from a normal donor were incubated with or without (A) AZD1283 (30 μ M) (B), U73122 (10 μ M), LY294002 (30 μ M), GF109203X (30 μ M) and then stimulated with 160 μ M AYPGKF for additional 15 min. Platelets were labeled with FITC-Pac1 Ab. P-selectin expression: platelets from a normal donor were incubated with or without AZD1283 (30 μ M) (C), U73122 (10 μ M), LY294002 (30 μ M), GF109203X (30 μ M) (D) and then stimulated with 160 μ M AYPGKF for additional 10 min. Platelets were labeled with PE-P-Selectin Ab. Representative data are presented from 3 separate experiments. (E) PAR4 mediated Rap1 activation in human platelets from PAR4 variant rs773902 (A/ A homozygote Ala/Ala120 PAR4, T/A heterozygote Thr120/Ala120 PAR4 and T/T homozygote Thr/Thr120 PAR4). Human platelets were stimulated with 160 μ M AYPGKF for the indicated times in minutes, and active Rap1-GTP was detected by pull-down assay. Total Rap1 is shown as loading control. Effect of pretreatment with AZD1283 (30 μ M) on

PAR4 (160 μ M AYPGKF) activation on human platelets was determined. Representative data is presented from n = 6, 3 AA, 2 TA and one TT individual in duplicate.

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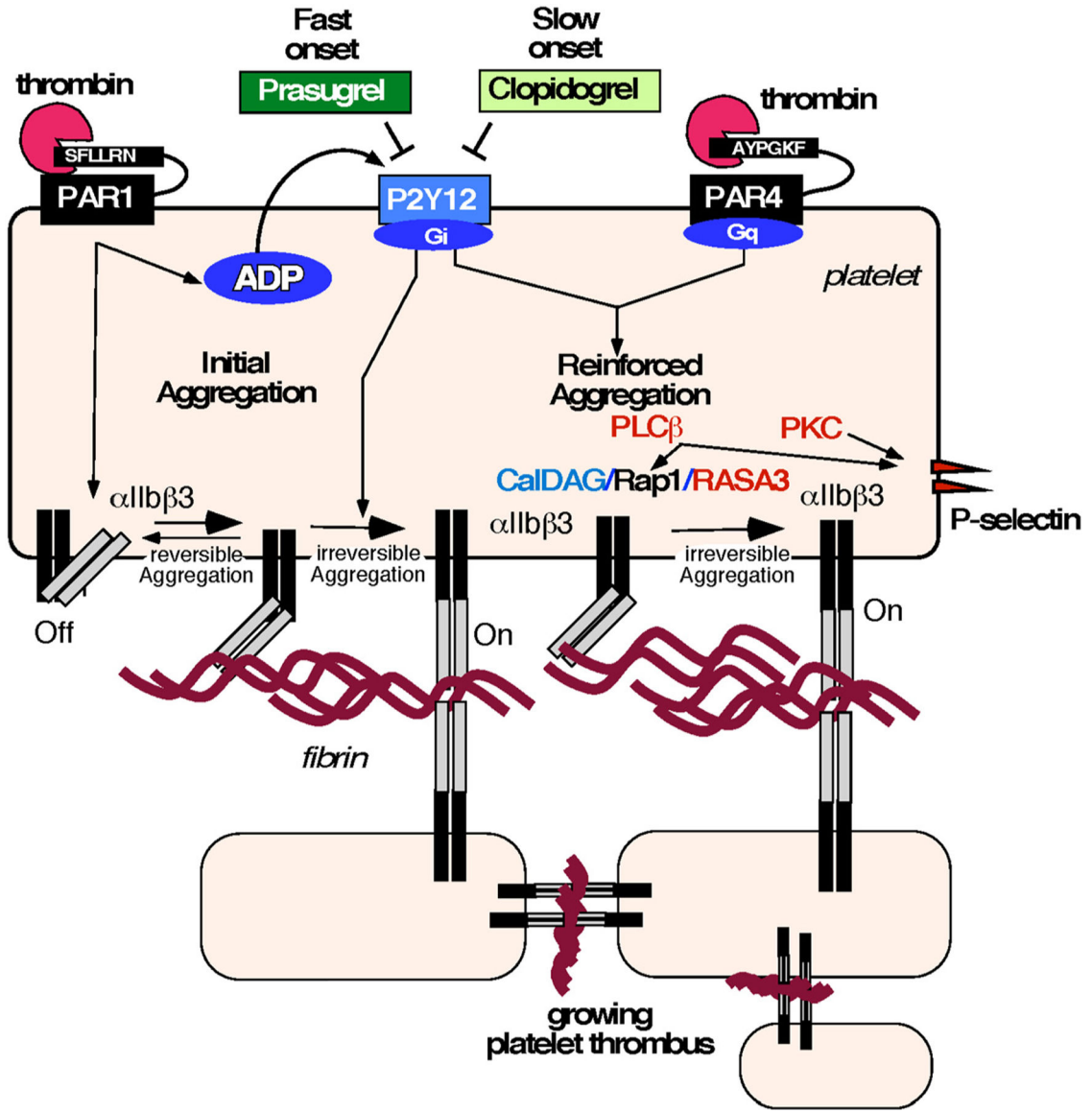


Fig. 8. Mechanism of PAR4/P2Y₁₂ mediated α IIb β 3 and Rap1 activation and P-selectin expression. Schematic model of platelet activation upon prasugrel-mediated reduction in stent thrombosis through inhibition of dual thrombin receptors. Fast onset of inhibition of ADP signaling with the enhanced P2Y₁₂ inhibitor prasugrel uncovered a new role for P2Y₁₂/PAR4 in formation of more stable, growing platelet aggregates which contribute to both enhanced hemostasis and thrombosis.

Table 1

PCI Patients, demographics and clinical outcome.

	Clopidogrel	Prasugrel
Patients(n)	12	12
Age (years)	64 ± 8	59 ± 9
Female/Male	4/8	1/11
Race		
Caucasian	10	11
Asian	2	1
Weight (kg)	88 ± 20	90 ± 14
Hemoglobin (g/dL)	13.8 ± 1.7	14.4 ± 1.2
Creatinine(mg/dL)	0.93 ± 0.19	1.04 ± 0.28
Platelets (k/ μ L)	219 ± 50	211 ± 46
Stent deployed (#patients)	12	12
Smokers (n, %)		
Current	1 (8.3%)	1 (8.3%)
Former	7 (58%)	7 (58%)
Urgent reocclusion	None	None
Cardiac by-pass surgery	None	None
Myocardial infarction	None	None
Death	None	None
Minor or major hemorrhage	None	None

Data are reported as Mean ± SD.

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Table 2

Inhibitory effects of clopidogrel (n = 12) and Prasugrel (n = 12) on platelet aggregation and systemic TAT levels in PCI patients.

Agonist	Time (hour)	Clopidogrel inhibition (% ± SE)	Prasugrel inhibition (% ± SE)	p-value
ADP 20 μM	1	38 ± 10	84 ± 9	< 0.0001
	2	53 ± 10	94 ± 5	
	4	72 ± 8	98 ± 1	
	16	85 ± 3	98 ± 1	
SFLLRN 5 μM	1	2 ± 8	62 ± 9	< 0.0001
	2	9 ± 8	64 ± 10	
	4	17 ± 8	75 ± 6	
	16	15 ± 8	58 ± 7	
AYPGKF 160 μM	1	7 ± 3	39 ± 9	< 0.0001
	2	9 ± 3	44 ± 11	
	4	13 ± 4	53 ± 8	
	16	11 ± 4	41 ± 10	
Collagen ^a 5 μg/mL	1	34 ± 11	56 ± 8	0.06
	2	39 ± 9	61 ± 6	
	4	58 ± 8	67 ± 4	
	16	48 ± 8	61 ± 8	
TAT pmol/L	0	258 ± 39	251 ± 27	NS
	1	274 ± 82	179 ± 23	NS
	2	180 ± 23	226 ± 35	NS
	4	284 ± 34	224 ± 15	NS
	16	151 ± 10	301 ± 45	0.007

Platelets in PRP were isolated from PCI patients at baseline and after 1, 2, 4 and 16 h after bivalirudin infusion and clopidogrel and prasugrel loading dose administration, and were stimulated with 20 μM ADP, 5 μM SFLLRN, 160 μM AYPGKF and 5 μg/L collagen. TAT levels in plasma were analyzed by ELISA as described in the methods. Data are reported as Mean ± SD and p values were determined by one-way ANOVA repeated measure test. TAT statistical analyses were performed using two-tailed student's *t*-test.

^aCollagen-dependent aggregation was measured with 18 of the 24 PCI patients as shown in Fig. 4A for individual patients.