Inhibitory Effects of P2Y12 Receptor Antagonist on PAR1- and PAR4-AP-Induced Platelet Aggregation in Patients with Stroke or TIA

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Objectives: The inhibitory effects of P2Y12 receptor antagonist on PAR1- and PAR4-activating peptide (AP)-induced platelet aggregation have not been fully elucidated. The present study aimed to investigate the inhibitory effects of P2Y12 receptor antagonist on PAR1- and PAR4-AP-induced platelet aggregation using platelet-rich plasma (PRP) from individuals including patients with stroke or transient ischemic attack (TIA). *Materials and Methods:* PRP was given to 10 healthy individuals pretreated in vitro with cangrelor, then stimulated with adenosine diphosphate (ADP), PAR4-AP, or PAR1-AP. Moreover, 20 patients were enrolled from 148 consecutive patients with acute ischemic stroke or TIA admitted to our institute between December 2017 and April 2019. PRP obtained from each patient before and >7 days after initiation of clopidogrel was similarly stimulated with these agonists. Platelet aggregation was measured using an automatic coagulation analyzer in all participants. *Results:* In healthy individuals, ADP- and PAR4-AP-induced platelet aggregations were significantly inhibited depending on the cangrelor concentration in vitro, while PAR1-AP-induced platelet aggregation was slightly inhibited. In patients with stroke or TIA, clopidogrel inhibited ADP-induced platelet aggregation at all concentrations, and significantly inhibited PAR4-AP-induced platelet aggregation at 50 μmol/L of PAR4-AP ($p<0.05$), especially in 5 patients who showed high reactivity to PAR4-AP. PAR1-AP-induced platelet aggregation was also slightly inhibited. *Conclusions:* We showed significant inhibitory effects on PAR4-AP-induced platelet aggregation by clopidogrel in patients with stroke or TIA who had high reactivity to PAR4-AP.

Key Words: Stroke—Platelet aggregation—PAR4—PAR1—P2Y12 receptor antagonist—Clopidogrel

Introduction

Platelet aggregation is a key stage in the formation of arterial thrombi, which cause thrombotic events such as stroke or acute coronary syndrome, and is thus employed to evaluate platelet function and reactivity.[1-3] Individual differences in platelet aggregation have been reported.[4-10] Ethnic differences in platelet aggregation could explain differences in the incidences and outcomes of ischemic heart disease,[2] but platelet aggregation has been associated with coronary heart disease in some epidemiological studies,[3]

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whereas not in others.[4] A systematic review of the literature concluded that evaluation of platelet function/reactivity was not justified as a basis for altering antiplatelet therapy in individuals on secondary stroke prevention in routine clinical practice.[1] Although many agonists, including ADP, arachidonic acid, epinephrine, and collagen, have been studied in the literature, thrombin has not received attention as a platelet-activating agonist.

Recently, thrombin has been reported as the most potent activator of platelets.[11,12] Thrombin activates human platelets through protease-activated receptor (PAR),[13] a unique family of seven transmembrane domain receptors, G-protein-coupled receptors, characterized by self-activating mechanisms. Human platelets express two thrombin receptors, PAR1 and PAR4.[13] PAR1 shows higher affinity for thrombin and mediates rapid Ca^{2+} mobilization and platelet activation. By contrast, PAR4 activation requires higher concentrations of thrombin and leads to slower but more sustained Ca^{2+} mobilization, which may be necessary for stable platelet thrombus formation.[14,15] PAR4 has the ability to form homo-oligomers with itself, and hetero-oligomers with PAR1 and P2Y12 receptor.[14-19] Thrombin-mediated PAR1-PAR4 activation and other G-protein-coupled receptors such as P2Y12 thus play collaborative roles in platelet activation and the signaling that controls thrombosis and hemostasis.[20]

The role of PAR1-AP-induced platelet aggregation has been addressed, and a PAR1 antagonist, vorapaxar, was clinically studied in patients with coronary heart disease and peripheral artery disease.[21] However, PAR4-induced platelet aggregation has not been fully addressed in clinical settings. Previous studies have assessed the inhibitory effects of P2Y12 receptor antagonist on PAR4-AP-induced platelet aggregation in vitro using washed platelets from healthy individuals.[17] Inhibitory effects on PAR4-AP-induced platelet aggregation from prasugrel in patients receiving percutaneous coronary intervention have been reported.[20] On the other hand, the inhibitory effects on PAR1- and PAR4-AP-induced platelet aggregation of P2Y12 receptor antagonists in patients with stroke or transient ischemic attack (TIA) have not been studied, particularly in settings using platelet-rich plasma (PRP). The aim of the present study was to investigate the inhibitory effects of P2Y12 receptor antagonists, including clopidogrel and in-vitro cangrelor, on PAR1- and PAR4-AP-induced platelet aggregation using PRP from individuals including patients with stroke or TIA.

Methods

Subjects

This was a single-center, prospective study. First, in-vitro assay of healthy individuals was performed to confirm the inhibitory effects of P2Y12 receptor antagonist on PAR4-AP-induced platelet aggregation using PRP. In 2017, we recruited 10 healthy volunteers (5 men; median age, 33 years; range, 26−52 years) who had no physical signs or symptoms of disease and were not taking any medications. For the subsequent clinical study to evaluate patients with stroke or TIA treated by P2Y12 receptor antagonist, we screened 148 consecutive patients with acute ischemic stroke or TIA who were admitted to Iwate Medical University Hospital (Iwate, Japan) during office hours (08:00−17:00, Monday−Friday) between December 2017 and April 2019. Among these, we enrolled patients who could underwent platelet aggregation testing within 2 h from admission. Based on the findings from clinical and brain imaging, board-certified stroke neurologists made a diagnosis of ischemic stroke or TIA. Exclusion criteria were defined as follows: 1) treatment with P2Y12 antagonists before index stroke onset; 2) meeting indications for anticoagulation therapy such as atrial fibrillation, prosthetic valve, or venous thromboembolism; 3) meeting contraindications for clopidogrel; 4) active bleeding or high risk of bleeding; 5) platelet count < 150,000/μL; 6) severe dysphasia; 7) malignant tumor; 8) severe hepatic failure; or 9) other circumstances considered inappropriate by the investigators. The following background characteristics were investigated: age, sex, body mass index, hypertension (blood pressure ≥ 140/90 mmHg before stroke onset or current antihypertensive medication), diabetes mellitus (fasting blood glucose ≥ 126 mg/dL, random blood glucose ≥ 200 mg/dL, hemoglobin A1c ≥ 6.5%, or current antidiabetic medication), dyslipidemia (serum total cholesterol ≥ 220 mg/dL, triglycerides > 150 mg/dL, or current antihyperlipidemic medication), liver dysfunction, current smoking habit, history of myocardial infarction or stroke, and oral medication including oral proton pump inhibitors.

All participants provided written informed consent prior to collection of samples. The present study was approved by the institutional ethics committee and conducted in accordance with the Declaration of Helsinki.

Materials

Sample collection

In all participants, blood samples were obtained from an antecubital vein using a 21-G needle. In patients with stroke or TIA, blood samples from patients were collected twice: just before clopidogrel treatment; and >7 days after treatment. The first 2 mL of blood was discarded, and the remaining volume was collected in a tube with 3.13% sodium citrate for aggregation testing. PRP was separated by low-speed centrifugation according to the previously described protocol.[22,23] Briefly, venous blood was centrifuged at 75 × *g* for 15 min to obtain PRP. Platelet-poor plasma (PPP) was obtained by centrifugation of blood from which PRP had been removed at 1500 × *g* for 5 min. PRP samples were used without dilution with autologous PPP in all controls and patients.

Measurement and analysis of platelet aggregation

Platelet aggregation was determined by measuring light transmission using a CS-2400 automated platelet

aggregometer (Sysmex, Kobe, Japan), a light transmission aggregometry system developed on a routine coagulation analyzer.[24] Platelet aggregation was calculated based on the percentage maximum platelet aggregation (MA%), determined by measuring the reaction for 420 s after addition of each agonist. All studies were performed within 2 h after blood collection.

ADP-, PAR4-AP-, and PAR1-AP-induced platelet aggregations were measured under stirring conditions using PRP. Cangrelor (Cangrelor; Cayman Chemical, Ann Arbor, MI) was employed as the P2Y12 receptor antagonist for in-vitro assay in healthy individuals, because this is an active drug for intravenous application. We conducted a series of experiments with PAR-activating peptides (AP) that have been extensively used to describe the signaling of PARs.[17] For the in-vitro study, we measured platelet aggregation in PRP from 10 healthy volunteers with 10 μmol/L of ADP (Revohem ADP; Sysmex, Kobe, Japan), 100 μmol/L of PAR4-AP (AYPGKF-NH2; GenScript, Piscataway, NJ), and 10 μmol/L of PAR1-AP (SFLLRN-NH2; Bachem, Bubendorf, Switzerland), before and after pretreatment with each concentration of cangrelor (0.04, 0.2, 0.4, 1, 2, 4, 8, 20, and 40 nmol/L). In patients with acute non-cardioembolic stroke or TIA, we also measured platelet aggregation in the range of 0.25−20 μmol/L of ADP, 25−200 μmol/L of PAR4-AP, and 0.1−10 μmol/L of PAR1-AP, at two time points, pre-treatment and >7 days after clopidogrel treatment.

Statistical analysis

All data were statistically analyzed using SPSS version 25 software (IBM Japan, Tokyo, Japan). In the in-vitro study of blood from health individuals, ADP-, PAR4-AP- and PAR1-AP-induced platelet aggregations were compared between samples without cangrelor and with each concentration of cangrelor, respectively, using the Wilcoxon signed-rank test. In patients with stroke and TIA, ADP-, PAR4-AP-, and PAR1-AP-induced platelet aggregations for each concentration were compared between samples collected before and after clopidogrel administration using the Wilcoxon signed-rank test. Values of $P<0.05$ were considered statistically significant.

Results

Effects of cangrelor in vitro on platelets from healthy subjects

We evaluated platelet aggregation in PRP from 10 healthy individuals.

ADP-induced platelet aggregation

Median MA% on ADP-induced platelet aggregation was 83.5% (interquartile range (IQR), 76.9−86.5%) before pretreatment. Cangrelor significantly inhibited ADP-induced platelet aggregation at all concentrations (0.04−40 nmol/L, $p<0.01$) (Fig. 1A).

PAR4-AP-induced platelet aggregation

Cangrelor significantly inhibited PAR4-AP-induced platelet aggregation at all concentrations (0.04−40 nmol/L, $p<0.01$) (Fig. 1B). Median MA% on PAR4-AP-induced platelet aggregation was 91.3% (IQR, 90.3−92.6%) before pretreatment. Median MA% with each concentration of cangrelor (0.04−40 nmol/L) was 75.8% (IQR, 63.7−84.4%), 64.0% (50.8−75.9%), 61.7% (53.9−74.8%), 65.1% (54.9−77.7%), 63.1% (55.7−79.8%), 61.5% (54.0−76.9%), 63.5% (51.1−75.0%), 66.5% (54.8−79.9%), and 65.2% (54.5−81.1%), respectively. No sex differences were apparent in PAR4-AP-induced platelet aggregation.

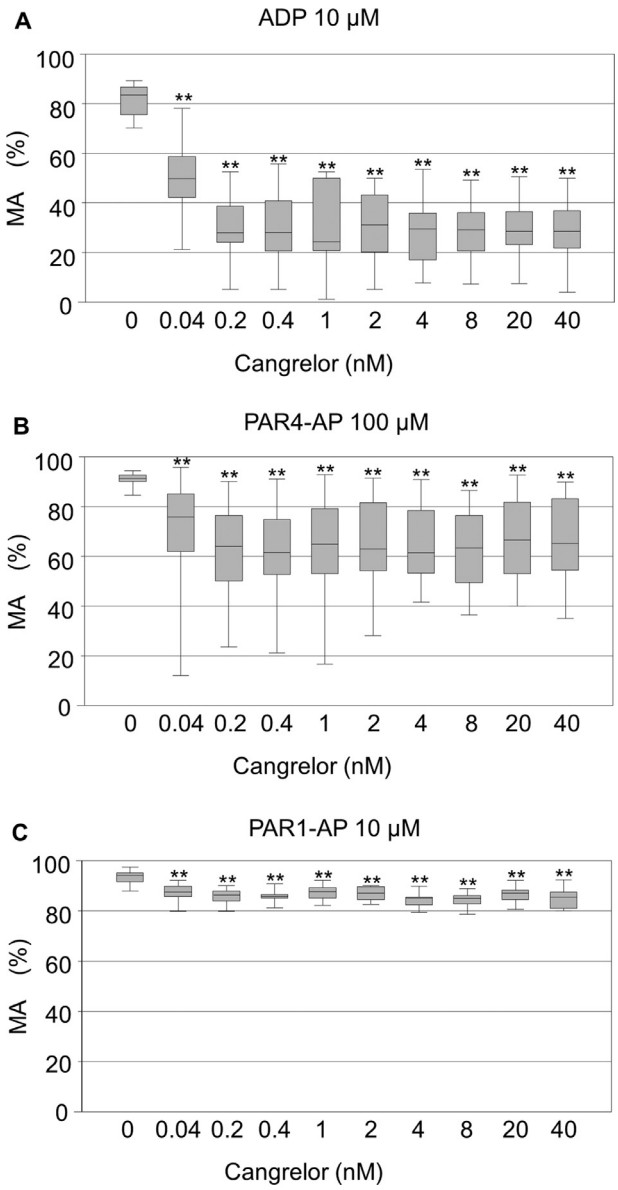

Fig. 1. *Antiplatelet effects of cangrelor on platelets from healthy subjects. Data from 10 healthy subjects are presented. ADP- and PAR4-AP-induced platelet aggregation were inhibited at all concentrations (**A, B**). PAR1-AP-induced platelet aggregation was slightly inhibited (**C**). Data are presented as scatter diagrams and box plots. Differences between before and after addition of cangrelor (0.04−40 nmol/L) were examined using the Wilcoxon signed-rank test. Values of* P*<0.05 were considered significant. ** p<0.01*

PAR1-AP-induced platelet aggregation

Cangrelor slightly but significantly inhibited PAR1-AP-induced platelet aggregation at all concentrations (0.04−40 nmol/L, $p<0.01$) (Fig. 1C). Median MA% on PAR1-AP-induced platelet aggregation was 94.1% (IQR, 92.1−94.9%) before pretreatment. Median MA% with each concentration of cangrelor (0.04−40 nmol/L) was 87.4% (IQR, 85.6−89.5%), 86.3% (84.0−87.6%), 85.6% (85.1−86.2%), 87.6% (85.2−89.0%), 87.1% (84.6−89.3%), 85.0% (82.7−85.5%), 84.9% (82.9−85.8%), 87.0% (84.9−88.0%), and 85.5% (81.9−87.2%), respectively. No sex differences were apparent in PAR1-AP-induced platelet aggregation.

Effects of clopidogrel on platelets from patients with ischemic stroke or TIA

Finally, we prospectively enrolled 20 patients (10 men; median age, 67 years; range, 47−88 years) into this study. These comprised 17 patients with acute ischemic stroke and 3 patients with TIA. There were no patients who received antiplatelet drug including aspirin before stroke onset. Median time from stroke onset to first measurement of platelet aggregation as pre-treatment with clopidogrel and from start of clopidogrel to second measurement of platelet aggregation as post-treatment was 0 days (IQR, 0−2.5 days) and 14 days (IQR, 10.5−20.3 days), respectively. Among these 20 patients, 16 patients were prescribed clopidogrel at 75 mg once daily, and 4 patients were prescribed clopidogrel at 75 mg and aspirin at 100 mg once a day. No patients were started with a loading dose such as clopidogrel at 300 mg. Baseline characteristics of patients are listed in Table 1.

ADP-induced platelet aggregation

ADP-induced platelet aggregation was elevated in a concentration-dependent manner before treatment with clopidogrel (Fig. 2A). In samples collected after treatment with clopidogrel, ADP-induced platelet aggregation was significantly inhibited at all concentrations (0.25 μmol/L, $p<0.05$; 1−20 μmol/L, $p<0.001$) (Fig. 2A).

Table 1. *Characteristics of patients.*

Characteristics	*n*=20
Age, years (mean ± SD)	71 ± 12
Male sex, *n* (%)	10 (50%)
Body mass index, kg/m^2 (mean ± SD)	23.6 ± 4.7
Hypertension, *n* (%)	15 (75%)
Diabetes mellitus, *n* (%)	7 (35%)
Dyslipidemia, *n* (%)	12 (60%)
History of liver dysfunction	0 (0%)
Current smokers, *n* (%)	7 (35%)
History of myocardial infarction, *n* (%)	0 (0%)
History of ischemic stroke, *n* (%)	0 (0%)
Proton-pump inhibitor, *n* (%)	2 (10%)

SD, standard deviation; *n*, number.

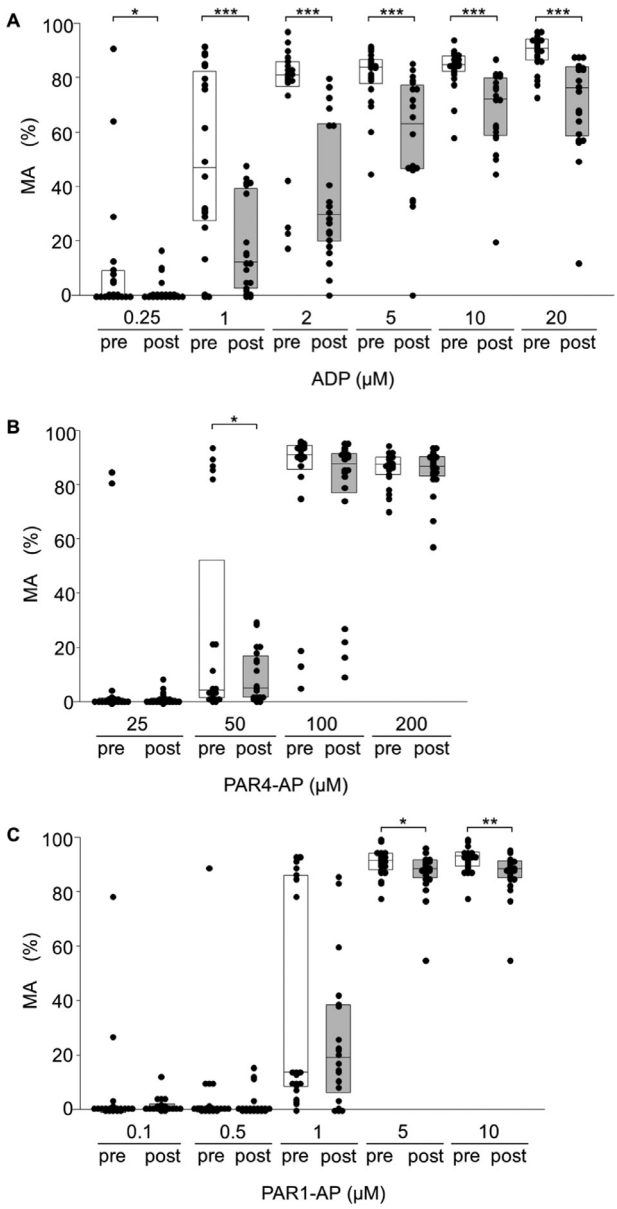

Fig. 2. *Antiplatelet effects of clopidogrel on platelets from ischemic stroke patients. Data from 20 patients with ischemic stroke are presented. ADP-induced platelet aggregation was markedly inhibited at all concentrations* ***(A)****. PAR4-AP-induced platelet aggregation was significantly inhibited at 50 μmol/L* ***(B)****. PAR1-AP-induced platelet aggregation was inhibited at 5 and 10 μmol/L* ***(C)****. Data are presented as scatter diagrams and box plots. Differences between before medication and >7 days after medication were examined using the Wilcoxon signed-rank test. Values of* $P < 0.05$ *were considered significant. * p<0.05; ** p<0.01; *** p<0.001*

PAR4-AP-induced platelet aggregation

Before treatment with clopidogrel, median MA% with 25, 50, 100, and 200 μmol/L of PAR4-AP was 0.6% (IQR, 0.4−1.0%), 4.4% (1.5−36.8%), 91.2% (86.6−94.4%), and 87.6% (84.0−89.8%), respectively. In samples collected after treatment with clopidogrel, PAR4-AP-induced platelet aggregation was significantly inhibited from 4.4% (IQR, 1.5−36.8%) to 5.0% (1.7−16.5%) at 50 μmol/L ($p<0.05$)

(Fig. 2B). At high concentrations of PAR4-AP (100 or 200 μmol/L) stimulation, PAR4-AP-induced platelet aggregation was not inhibited. Median MA% after treatment with clopidogrel was 0.6% (IQR, 0.3−1.2%), 5.0% (1.7−16.5%), 87.7% (78.2−91.4%), and 86.8% (83.6−90.4%), respectively. The significant change in platelet aggregation at 50 μmol/L of PAR4-AP was attributed to five patients who showed high reactivity to PAR4-AP stimulation before treatment (Fig. 2B). No sex differences were apparent in PAR4-AP-induced platelet aggregation.

PAR1-AP-induced platelet aggregation

Median MA% with 0.1, 0.5, 1, 5, and 10 μmol/L of PAR1-AP before treatment with clopidogrel was 0% (IQR, 0−0.6%), 0% (0−0.65%), 13.8% (8.9−85.8%), 91.6% (88.4−94.0%), and 93.2% (89.7−94.5%), respectively. In samples collected after treatment with clopidogrel, PAR1-AP-induced platelet aggregation was slightly but significantly inhibited at 5 μmol/L and 10 μmol/L (5 μmol/L, $p<0.05$; 10 μmol/L, $p<0.01$) (Fig. 2C). Median MA% after taking clopidogrel was 0.65% (IQR, 0−1.6%), 0% (0−0%), 19.2% (7.3−38.3%), 88.6% (85.2−91.7%), and 88.5% (85.2−91.0%), respectively. No sex differences were apparent in PAR1-AP-induced platelet aggregation.

Discussion

In this study, in addition to inhibition of ADP-induced platelet aggregation, we confirmed inhibitory effects on PAR4-AP-induced platelet aggregation from P2Y12 receptor antagonist with an in-vitro assay in healthy individuals. Moreover, we found that PAR4-AP-induced platelet aggregation was clearly inhibited by clopidogrel in patients with stroke or TIA who displayed high reactivity to PAR4-AP. We also found slight inhibitory effects on PAR1-AP-induced platelet aggregation by P2Y12 receptor antagonists both in healthy individuals and patients with stroke or TIA.

Holinstat et al. reported that PAR4-AP-mediated aggregation was severely attenuated following dual inhibition of calcium mobilization and P2Y12 receptor signaling using washed platelets in vitro.[17] Although our data were consistent with that previous study, we conducted all assays using PRP, not washed platelets. PRP is useful for creating a state close to the actual in-vivo environment.

We showed that PAR4-AP-induced platelet aggregation was inhibited in patients with high reactivity for PAR4-AP-induced platelet aggregation. Patients with ischemic stroke who showed high reactivity for PAR4-AP-induced platelet aggregation could be favorable candidates for receiving clopidogrel. Clopidogrel has been widely used as a P2Y12 antagonist for secondary prevention in patients with ischemic stroke in the chronic setting.[25] Furthermore, dual antiplatelet therapy with aspirin and clopidogrel is recommended for acute high-risk TIA and minor ischemic stroke to reduce the risk of recurrent stroke or death.[26] Individual differences in the antiplatelet effects of clopidogrel have been largely attributed to genetic variations in cytochrome P450 (CYP)2C19, as the major enzyme involved in the generation of the active metabolite of clopidogrel.[27] However, PAR4-AP-induced platelet aggregation could explain part of the individual differences in response to P2Y12 inhibitor. PAR4-AP-induced platelet aggregation before receiving clopidogrel reportedly differs from individual to individual.[4-10] Moreover, PAR4-induced platelet aggregation differs by ethnic background due to single nucleotide polymorphisms.[4-10] Morikawa et al. reported that PAR4 gene F2RL3 variants influence platelet reactivity induced by PAR4-AP.[10] Although the association between PAR4-AP-induced platelet aggregation and risk of cardiovascular disease remains unclear, the rs773902 A allele was reportedly associated with PAR4-AP-induced platelet function and risk of stroke.[8]

We showed slight inhibitory effects on PAR1-AP-induced platelet aggregation from P2Y12 receptor antagonists in both healthy individuals and patients with stroke or TIA. PAR1-AP-induced platelet aggregation might be inhibited through suppression of autocrine ADP release by cangrelor.[28] Kimmelstiel et al. also reported inhibition of PAR1-induced platelet aggregation by P2Y12 antagonist. Initial platelet generation of thrombin and binding to PAR1 results in PAR1 activation and release of dense granules containing ADP. ADP-mediated activation of P2Y12 further activates platelets in synergy with PAR1, resulting in irreversible platelet aggregation.[20]

More potent platelet P2Y12 antagonists, such as prasugrel and ticagrelor, are preferred over clopidogrel in patients with coronary heart disease undergoing percutaneous coronary intervention.[29] Kimmelstiel et al. reported that prasugrel has greater effects than clopidogrel on the inhibition of platelet aggregation, including inhibition of the PAR4 receptor.[20] Wadowski et al. reported that ticagrelor could inhibit PAR4-mediated platelet activation more strongly than prasugrel in patients with acute coronary syndrome.[30] Those two novel P2Y12 antagonists failed to show more significant effects on prevention of cardiovascular events than conventional antiplatelet agents in patients with ischemic stroke or TIA.[31,32] However, it was just recently reported that ticagrelor additional to aspirin was effective for the acute treatment of mild stroke or TIA.[33]

Our study has several limitations. First, we might have underestimated differences between groups because of the small sample size. Second, we could not perform genetic analyses to explain the differences in response to platelet activation among healthy individuals and patients. Third, we did not study genetic variants of CYP2C19; however, ADP-mediated platelet aggregation was well inhibited in all subjects. We think that the effect of CYP2C19 on drug metabolism was limited in our study. Fourth, we evaluated platelet function several weeks after onset and initiation of antiplatelet treatment, so we could not perform long-term evaluations in patients with ischemic stroke.

In conclusion, we showed significant inhibitory effects on PAR4-AP-induced platelet aggregation by clopidogrel in patients with stroke or TIA who had high reactivity to PAR4-AP. Our data indicated that patients with ischemic stroke who showed high reactivity for PAR4-AP-induced platelet aggregation could be favorable candidates for antiplatelet treatment using P2Y12 receptor antagonists. To address associations between PAR4 and P2Y12 receptor antagonists in greater detail and more robustly, a large, prospective registry is warranted.

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Declaration of Competing Interest

The Authors declare that there is no conflict of interest.

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