Functional Significance of Adenosine 5′-Diphosphate Receptor (P2Y₁₂) in Platelet Activation Initiated by Binding of von Willebrand Factor to Platelet GP Ibα Induced by Conditions of High Shear Rate

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Background—The role of the adenosine 5′-diphosphate receptor P2Y₁₂ in platelet activation initiated by the von Willebrand factor (VWF)-GP Ibα interaction under high shear rate was investigated.

Methods and Results—Blood samples were obtained from 11 donors. Shear-induced platelet aggregation was detected by optically modified cone-plate viscometer. Shear-induced VWF binding, P-selectin expression, and microparticle release were detected by flow cytometry. Platelet interaction with immobilized VWF was also investigated by parallel-plate flow chamber equipped with epifluorescent videomicroscopy. Effects of a selective P2Y₁₂ antagonist AR-C69931 MX were tested. AR-C69931 MX inhibited shear-induced platelet aggregation in a dose-dependent manner, achieving the maximum inhibition at 100 nmol/L. The extent of aggregation after exposure to a shear rate of 10 800 s⁻¹ for 6 minutes in the presence of 100 nmol/L AR-C69931 MX was 32.4±8.2% (mean±SD), which was significantly lower than the value in the controls of 69.7±9.6% (P<0.01). The inhibiting effects of AR-C69931 MX were reversed by exogenous addition of adenosine 5′-diphosphate. Shear-induced VWF binding and P-selectin surface translocation, which occurred in 4696±911 and 5964±784, respectively, of 10 000 measured platelets, was also inhibited by AR-C69931 MX (100 nmol/L) to 1948±528 and 2797±718, respectively (P=0.0018 and P=0.0009). Microparticle release was similarly inhibited. In a flow chamber experiment, firm platelet attachment on immobilized VWF was inhibited by AR-C69931 MX, whereas transient interaction was not influenced. All the above reactions were completely inhibited by blocking VWF–GP Ibα interaction.

Conclusions—We have demonstrated that the stimulation of P2Y₁₂ is involved in platelet activation initiated by the binding of VWF to GP Ibα induced by a high shear rate. (Circulation. 2002;105:2531-2536.)

Key Words: platelets ■ thrombosis ■ glycoproteins ■ von Willebrand factor ■ receptors

Recent investigations have revealed that the antiplatelet effects of thienopyridine antiplatelet agents such as ticlopidine and clopidogrel depend on the specific and irreversible inhibition of platelet adenosine 5′-diphosphate (ADP) receptors coupled with Gi/adenylate cyclase (P2TAC), recently cloned as P2Y₁₂, by their active metabolites. Although 3 different ADP receptors mediating different cellular reactions of platelets have been cloned so far (eg, P2X₁, which mediates calcium influx, P2Y₁, which mediates intracellular calcium mobilization, and P2Y₁₂, a Gi-coupled receptor mediating the action of adenylate cyclase and other more complex signaling that has yet to be clarified), understanding the role played by P2Y₁₂ receptors is particularly important since large-scale randomized clinical studies have shown the effectiveness of thienopyridine antiplatelet agents in preventing arterial thrombotic disease. Many reports indicate that P2Y₁₂ stimulation by exogenously added ADP is crucial for platelet activation and aggregation in vitro. However, the important issue—why in vivo arterial thrombosis was prevented by its inhibition—is still not fully understood.

Recent investigation revealed that von Willebrand factor (VWF) and its interaction with platelet receptor proteins GP Ibα and GP IIb/IIIa played an important role in the onset of platelet thrombosis at sites exposed to high shear rates. In this study, we attempted to clarify the role of the P2Y₁₂ receptor inhibition in VWF-mediated platelet activation and aggregation under high shear stress by using the specific P2Y₁₂ antagonist AR-C69931 MX.

Methods

Preparation of Platelet Samples
Platelet-rich plasma was separated by centrifugation of blood specimens anticoagulated by citrate (0.38%), obtained from healthy adult...
donors who abstained from drugs known to interfere with platelet function (such as nonsteroidal antiinflammatory drugs) for at least 4 weeks. Additional blood samples obtained for the flow experiments were anticoagulated by the specific antithrombin agent Argatroban (Mitsubishi Kagaku Co) at a final concentration of 100 μmol/L.

Shear-Induced Platelet Aggregation, VWF Binding to Platelets, and Platelet Activation
Platelet aggregation under a selected shear rate can be measured with the use of an optically modified cone-plate viscometer as described previously.14–16,17 Effects of AR-C69931 MX was tested at 10 800 s⁻¹ because a previous study demonstrated VWF binding with both GP Ibα and GP IIb/IIIa was required to induce aggregation at that high shear rate.17 Laser light transmittance of the sample was measured continuously while the samples were exposed to a shear field, and the measured light transmission, which was converted to the level of platelet aggregation based on the Lambert-Beer equation, was stored in a computer (PC-9800CX, NEC).17 Shear-induced VWF binding and subsequent activation with P-selectin surface translocation was detected by flow cytometry with FITC-conjugated specific anti-VWF (LJ-C3, provided by Dr Zaverio M. Ruggeri from the Scripps Research Institute)18 and anti–P-selectin (WGA1) monoclonal antibody.19 Two other agents, FITC-conjugated anti–GP Ibα (LJ-P3, also provided by Dr Zaverio)18 and FITC-conjugated Annexin V, which bind to phospholipid-rich procoagulant surfaces,20 were used to detect platelet-derived microparticles. LJ-P3 was used because it did not compete with VWF binding to GP Ibα.21 Shear-induced microparticle release was calculated as the difference between samples exposed to the shear forces and control samples that were not exposed.

Platelet Interaction With Immobilized VWF Under Flow Conditions
Human purified VWF was immobilized on glass coverslips (Corning, Inc; 24×50 mm) in a parallel-plate flow chamber, as described previously.22,23 Then, whole blood containing platelets rendered fluorescent by mepacrine was pumped through the chamber with a syringe pump at a constant flow rate to achieve a wall shear rate of 1500 s⁻¹. Platelets interacting with immobilized VWF were visualized under an inverted-stage epifluorescence videomicroscope equipped with a 480-nm excitation light source (DM IRB, 1RB-FLUO, Leica).24 The microscopic images were digitized on-line with a photosensitive color CCD camera (L-600, Leica) and stored as digital images in a personal computer (Power Macintosh G3, Apple Co, Ltd). The effects of AR-C69931 MX on platelet-VWF surface interaction was qualitatively demonstrated by overlaying 60 consecutive frames of the video images (corresponding to 2 seconds), so that firmly attached platelets that did not move appeared as thick images, whereas moving (eg, rolling) platelets, which appeared in only some of the frames, appeared as thinner images. The movement of every single platelet appearing in the consecutive image frames was quantified.

Reagents Used in Experiments
AR-C69931 MX, a specific inhibitor of P2Y₁₂, was kindly provided by AstraZeneca R&D Charnwood (Loughborough, Leicestershire, England). The basic characteristics of the material have been described elsewhere.15 FITC-conjugated PAC-1 and its binding to ADP-activated GP IIb/IIIa were detected by flow cytometry.24 This assay was used to characterize AR-C69931 MX. LJ-Ib1, the antibody blocking the binding of VWF to GP Ibα, was also kindly provided by Dr Zaverio. This antibody is known to block shear-induced binding of VWF to GP Ibα to generate a negative control.18

Statistical Analysis
All numerical data are expressed as mean±SD unless otherwise specified. The effect of various concentrations of AR-C69931 MX on platelet aggregation and activation was tested by 1-way ANOVA. Differences between two groups of data were compared by Fisher analysis. Statistical significance of the difference between two groups of data was tested by Student’s paired t test. A value of P<0.05 was considered to be statistically significant.

Results
Effects of AR-C69931 MX on Shear-Induced Platelet Aggregation
AR-C69931 MX inhibited shear-induced platelet aggregation in a dose-dependent manner at concentrations >5 nmol/L (Figure 1), with the maximum inhibition achieved at 100 nmol/L. The extent of aggregation after exposure to a shear rate of 10 800 s⁻¹ for 6 minutes in the presence of 100 nmol/L AR-C69931 MX was 32.4±8.2% (mean±SD), which was significantly lower than that in the absence of the agent of 69.7±9.6% (P<0.01). At relatively low concentrations, that is, <1 nmol/L, diaaggregation occurred, although the maximum extent of aggregation was not influenced. The aggregation observed under the tested shear rate was mediated by the interaction of VWF with GP Ibα, since it was completely inhibited by an antibody that would block this interaction.

Figure 1. Inhibiting effects of AR-C69931 MX on shear-induced platelet aggregation. To test the effects of the specific P2Y₁₂ antagonist AR-C69931 MX on shear-induced platelet aggregation, 400 μL of platelet-rich plasma was mixed with 100 μL of modified HEPES-NaCl solution (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4) containing various concentrations of AR-C69931 MX to achieve the final concentrations indicated. The same volume of HEPES-NaCl solution containing the anti-GP Ibα antibody (LJ-Ib1) at a concentration of 500 μg/mL was also added to 400 μL of platelet-rich plasma to test whether the aggregation under the shear rate tested were dependent on the VWF-GP Ibα interaction. a. Extent of platelet aggregation after 6 minutes’ exposure to a shear rate of 10 800 s⁻¹ at 25°C in the presence of various concentrations of AR-C69931 MX is shown. Result shown is from one series of representative experiments of 11 performed. b. Maximum extent of the platelet aggregation in the presence of various concentrations of AR-C69931 MX are summarized. Result shown are mean±SEM of 11 experiments. *P<0.05, **P<0.01.
(LJ-Ib1) (Figure 1). The inhibiting effect of AR-C69931 MX on the shear-induced aggregation was mediated by its competition for the P2Y12 receptor with ADP, since its inhibiting effects could be reversed by exogenous addition of ADP, as shown in Figure 2. Receptor competition between ADP and AR-C69931 MX was confirmed by a simple ADP-induced platelet activation assay, as shown in Figure 3. The inhibitory effects of AR-C69931 MX on ADP-induced platelet activation, as evidenced by the PAC-1 binding, was overcome by addition of extremely high concentrations of ADP.

**Effects of AR-C69931 MX on Shear-Induced Platelet Activation**

Of 10 000 platelets measured, shear-induced VWF binding and P-selectin translocation occurred in 4296±911 and 5964±784 platelets, respectively (Figure 4). Mean numbers of anti-VWF and anti-P-selectin antibodies binding to single platelets increased from 294.6±49.1 per platelet and 43.4±46.0 per platelet before shearing to 1369.4±318.8 per platelet and 1821±814 per platelet after shearing, respectively (P<0.0015 and P<0.0071, respectively). Platelet-derived microparticles, measured as particles with GP Ib/IIIa, which can bind PAC-1, was detected by flow cytometry and shown as arbitrary mean fluorescent intensity (MFI). PAC-1 binding after incubation with different concentrations of ADP in the absence or presence of AR-C69931 MX is shown. Results shown are mean±SEM of 11 experiments. *P<0.05, **P<0.01.

**Effects of AR-C69931 MX on Platelet Interaction With Immobilized VWF in Whole Blood Experiments**

Firm platelet attachment on immobilized VWF occurring under a wall shear rate of 1500 s⁻¹ was inhibited by AR-C69931 MX at a dose maximally inhibiting shear-
induced platelet aggregation (100 nmol/L), whereas temporally tethering and rolling on VWF surface was not inhibited (Figure 6). Platelets once adhered on the VWF surface in the absence of AR-C69931 MX moved only 0.38±0.35 μm during 2 seconds, whereas they moved 1.64±1.27 μm in the presence of 100 nmol/L AR-C69931 MX (P=0.0021).

**Discussion**

We have demonstrated that >50% of platelet activation and aggregation caused by the interaction of VWF with platelet GP Ibα under conditions of high shear rate in platelet-rich plasma could be inhibited by blocking the action of P2Y12 by AR-C69931 MX. We also demonstrated that firm platelet attachment on immobilized VWF in whole blood, presumably mediated by VWF binding to activated GP IIb/IIIa, is inhibited by AR-C69931 MX, suggesting the role of P2Y12 in VWF-mediated platelet activation in whole blood, too. These results, along with previously published findings demonstrating the involvement of VWF-mediated platelet thrombosis in animal arterial thrombosis models12,13 or ex vivo human studies,14,16–19,25–28 strongly suggest the importance of P2Y12 stimulation in the process of arterial thrombosis. Our present findings provide experimental evidence explaining the mechanism of in vivo antithrombotic action of P2Y12 inhibition achieved by thienopyridine antiplatelet agents such as ticlopidine and clopidogrel in the absence of exogenous addition of ADP. In addition, our results provide important experimental evidence showing that blocking P2Y12 stimulation by ADP released from platelets abrogates >50% of platelet reaction initiated by VWF–GP Ibα interaction.

Our findings with the specific P2Y12 antagonist AR-C69931 MX on shear-induced platelet activation and aggregation agree with previously published findings describing...
The effects of thienopyridines on shear-induced platelet aggregation and activation have been extensively studied,
and with recently published results with the same P2Y12 antagonist. However, new and important information regarding the
concentration-dependent effects of P2Y12 inhibition on shear-induced platelet reactions has ultimately been addressed in our study. Indeed, our results provide a possible explanation as to why dissociation of
once-aggregated platelets under high shear rates occurs after administration of ticlopidine,
whereas the maximum extent of shear-induced platelet aggregation remains uninfluenced. Partial P2Y12 inhibition may cause VWF-mediated aggregation less stable. At relatively high doses (>5 nmol/L),
dose-dependent and saturating effects of AR-C69931 MX against these agonists of ADP, as well as the inhibition of ADP-induced platelet aggregation, could be inhibited by a saturating dose of AR-C69931 MX that
approximately half of the platelet reactions initiated by the VWF-GP Ibα interaction depends on the enhancing role of
P2Y12 stimulation by ADP released from the platelets. Our results are in agreement with the recently published findings
of Turner et al., although the latter have also measured stable aggregates present when the shear stress was no longer
applied and tested only the highest concentration of AR-C69931 MX in their experiments. Our results, along with
previous publications demonstrating partial inhibition of collagen and thrombin-induced platelet activation by clopidogrel,
suggest an important role for platelet-derived ADP as a common enhancer of platelet activation.

There still are obvious methodological limitations in our experimental design that limit the application of our results to a
clearer understanding of the mechanism of the in vivo antithrombotic effects of thienopyridine antiplatelet agents.
Indeed, although the importance of the VWF-mediated mechanism of platelet thrombosis was demonstrated in animal
models of arterial thrombosis, the contribution of the VWF-mediated mechanism of platelet thrombus formation is
still speculative in the case of human arterial thrombosis. Thus, our idea that thienopyridine antiplatelet agents prevent
in vivo arterial thrombosis through inhibition of VWF-mediated platelet activation and aggregation is still speculative.
Further investigations will be required to demonstrate the role of VWF-mediated mechanisms in the process of in vivo arterial thrombosis. The other important limitation of our study is that the involvement of another important contribut-
ing factor for a thrombosis coagulant system was not considered. In the setting of thienopyridine antiplatelet therapy, in
addition to platelet thrombosis, fibrin formation might also be influenced, since we have demonstrated that platelet-derived microparticles, which are known to be a major source of platelet procoagulant activity, could be inhibited by P2Y12 inhibition with the use of AR-C69931 MX.

We used AR-C69931 MX as a specific P2Y12 antagonist. We have demonstrated the competitive effects of AR-C69931 MX and ADP on platelet activation, as evidenced by PAC1 binding, which detects activated GP IIb/IIIa.
Although at least 3 different subtypes of ADP receptors have been cloned, previous studies clearly indicate the nature of the
specific inhibition of P2Y12, by AR-C69931 MX, both through biochemical binding assays and by pharmacological studies.
AR-C69931 MX inhibits an ADP-induced decrease in cAMP but does not influence other cellular reaction induced by
ADP increases, such as Ca2+ influx, intracellular mobilization of Ca2+, or ADP-induced shape change in platelets. Those
results suggested that the effects of AR-C69931 MX on ADP receptors other than P2Y12 were negligible.

In conclusion, we demonstrated that stimulation of the P2Y12 receptor plays an important enhancing role in the
process of VWF-mediated platelet activation and aggregation occurring under high shear rates. Platelet-derived ADP and its
stimulation of P2Y12 may be a common enhancing system of the platelet response.

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