Adenosine Derived From ADP Can Contribute to Inhibition of Platelet Aggregation in the Presence of a P2Y\textsubscript{12} Antagonist

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Objective—To investigate whether adenosine diphosphate (ADP)–derived adenosine might inhibit platelet aggregation, especially in the presence of a P2Y\textsubscript{12} antagonist, where the effects of ADP at the P2Y\textsubscript{12} receptor would be prevented.

Methods and Results—Platelet aggregation was measured in response to thrombin receptor activator peptide by platelet counting in platelet-rich plasma (PRP) and whole blood in the presence of ADP and the P2Y\textsubscript{12} antagonists cangrelor, prasugrel active metabolite, and ticagrelor. In the presence of a P2Y\textsubscript{12} antagonist, preincubation of PRP with ADP inhibited aggregation; this effect was abolished by adenosine deaminase. No inhibition of aggregation occurred in whole blood except when dipyridamole was added to inhibit adenosine uptake into erythrocytes. The effects of ADP in PRP and whole blood were replicated using adenosine and were directly related to changes in cAMP (assessed by vasodilator-stimulated phosphoprotein phosphorylation). All results were the same irrespective of the P2Y\textsubscript{12} antagonist used.

Conclusion—ADP inhibits platelet aggregation in the presence of a P2Y\textsubscript{12} antagonist through conversion to adenosine. Inhibition occurs in PRP but not in whole blood except when adenosine uptake is inhibited. None of the P2Y\textsubscript{12} antagonists studied replicated the effects of dipyridamole in the experiments that were performed. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: antiplatelet drugs | arterial thrombosis | blood cells | cell physiology | g proteins | hemostasis | platelet receptor blockers | platelets | thrombosis | P2Y\textsubscript{12} antagonists | adenosine

ADP is a well-known platelet agonist that induces platelet aggregation. It is secreted from platelets themselves when they undergo a release reaction and potentiates the aggregatory effects of other platelet agonists, such as thrombin and collagen.\textsuperscript{1-3} The proaggregatory effects of ADP on platelets are via interaction with P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors,\textsuperscript{1-4} and one of the main effects of ADP at the P2Y\textsubscript{12} receptor is the inhibition of adenylate cyclase, leading to a reduction in cAMP levels and subsequent promotion of the aggregation response. Antagonists that act at P2Y\textsubscript{12} receptors markedly inhibit platelet aggregation, specifically by blocking the effects of ADP at this receptor,\textsuperscript{5} thereby preventing inhibition of adenylate cyclase. Such antagonists include cangrelor, ticagrelor, and the thienopyridines clopidogrel and prasugrel, all of which are in use or in development as antithrombotic agents.\textsuperscript{6-8}

Although ADP is conventionally regarded as a platelet agonist, the molecule can be broken down first to AMP and then to adenosine in blood and plasma.\textsuperscript{9-11} Adenosine, of course, is an inhibitor of platelet aggregation acting via A\textsubscript{2A} and A\textsubscript{2B} receptors on platelets to activate adenylate cyclase and increase cAMP,\textsuperscript{12-14} and both ADP removal and adenosine production might be expected to modulate platelet function.\textsuperscript{15-20} Such modulation may be particularly evident in the presence of a P2Y\textsubscript{12} antagonist that blocks the ability of the ADP to inhibit adenylate cyclase. There is already evidence, for example, that PGE\textsubscript{1} (which also inhibits platelet aggregation by activating adenylate cyclase) is a better inhibitor of platelet aggregation in the presence of cangrelor or clopidogrel\textsuperscript{21} and also that prostacyclin is a better inhibitor of platelet aggregation in the presence of cangrelor.\textsuperscript{22}

Another important consideration, however, is the fact that adenosine is readily removed from blood through uptake into red cells via the erythrocyte equilibrative nucleoside transporter,\textsuperscript{11,23,24} which would make the adenosine unavailable to interact with platelets and inhibit platelet aggregation. That is, unless uptake via the equilibrative nucleoside transporter is prevented in some way. It is known, for example, that dipyridamole is an inhibitor of adenosine uptake into red cells acting via the equilibrative nucleoside transporter.\textsuperscript{25-28} Also, the P2Y\textsubscript{12} antagonist ticagrelor has been reported to inhibit adenosine uptake into red cells, as well as acting on platelets via P2Y\textsubscript{12} antagonism, and might therefore allow adenosine to add to the effects of P2Y\textsubscript{12} blockade.\textsuperscript{20}
Against this background, we report here the results of a number of experiments that we have performed in both platelet-rich plasma (PRP; in which red cells are absent) and whole blood. We measured platelet aggregation induced by the thrombin receptor activator peptide (TRAP), which is a convenient means of mimicking the effects of thrombin on platelets, and also vasodilator-stimulated phosphoprotein (VASP) phosphorylation as a marker of cAMP. Our intentions were to determine the impact of any adenosine derived from ADP on the results obtained, especially in the presence of a P2Y12 antagonist and also to look at the importance of adenosine uptake by red cells in determining the outcome. The P2Y12 antagonists that were used in this investigation were cangrelor, prasugrel, and ticagrelor. Because prasugrel is a produg requiring metabolic conversion to generate the molecule that effects P2Y12 blockade, we used prasugrel active metabolite (PAM) in place of prasugrel in the experiments described here.

Materials and Methods

Materials

Hirudin (recombinant desulfatohirudin, Revasc) was a gift from Novartis (Basel, Switzerland). Cangrelor was a gift from The Medicines Company (Abingdon, United Kingdom) and was dissolved in saline. Ticagrelor and PAM were gifts from AstraZeneca (R&D Möln达尔, Möln达尔, Sweden) and were dissolved in dimethyl sulfoxide. TRAP, adenosine diphosphate (ADP), adenosine deaminase (ADA), adenosine, and dipyridamole were from Sigma Chemical Co (Poole, UK). Polytoser saline 0.9% was obtained from Fresenius Kabi Ltd (Chessley, UK). The fixative solution was 50 mmol/L sodium chloride containing 4.6 mmol/L EDTA, 4.5 mmol/L disodium hydrogen phosphate (Na2HPO4), 1.6 mmol/L potassium dihydrogen phosphate (KH2PO4), and 0.16% (wt/vol) formaldehyde, pH 7.4. For the VASP phosphorylation measurements, the Functional Bead Conjugation Buffer Set and Cell Signaling Master Buffer Kit were obtained from Becton Dickinson (Oxford, United Kingdom). IE273 anti-VASP was from Alexis Biochemicals (San Diego, Calif). Fluorescein isothiocyanate–conjugated antibody 5C6 anti-VASP pSer157 was from Acris Antibodies (Insight Biotechnology, Wembley, United Kingdom). Lysis buffer consisted of Tris-buffered saline, pH 7.6, from Sigma Chemical Co containing Triton X-100 (1%), the ionic detergent sodium deoxycholate (0.25%), and phenylmethylsulfonyl fluoride (1 mmol/L). A protease inhibitor tablet (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitor (Cocktail Set II, Calbiochem, Darmstadt, Germany) were also added to the lysis buffer according to the manufacturers’ instructions.

Methods

Blood Collection

Venous blood was obtained following informed consent from healthy volunteers who denied taking any medication in the preceding 14 days. Blood was taken into polystyrene tubes containing hirudin (final concentration, 50 μg/mL) and either incubated with the appropriate P2Y12 receptor antagonist (cangrelor [1 μmol/L], PAM [10 μmol/L], or ticagrelor [10 μmol/L]) at 37°C for 30 minutes before further experimentaion (whole blood studies) or immediately used to prepare PRP. To avoid any complications arising from the use of different vehicles for cangrelor, PAM, or ticagrelor, dimethyl sulfoxide was added to all samples. The concentration of dimethyl sulfoxide used (0.1%) had no effect on the degree of aggregation obtained.

Preparation of PRP

Freshly collected blood was centrifuged (180g, 10 minutes), and the supernatant PRP was removed. The remainder of the blood was recentrifuged (1500g, 10 minutes) to obtain platelet-poor plasma, and the PRP was diluted using autologous platelet-poor plasma to a standard platelet count of 300 × 10^9/L. The PRP was incubated with the appropriate P2Y12 receptor antagonist (cangrelor [1 μmol/L], PAM [10 μmol/L], or ticagrelor [10 μmol/L]) at room temperature for 30 minutes before further experimentation. Again, steps were taken to ensure that the concentration of dimethyl sulfoxide was always the same (0.1%).

Platelet Aggregation

Aliquots of PRP or whole blood (final volume, 495 μL) were dispensed into prewarmed polystyrene tubes (64×11 mm) containing the agent(s) under investigation (ADP [1, 3, and 10 μmol/L], adenosine [3 μmol/L], dipyridamole [10 μmol/L], and ADA [1.2U/mL] as appropriate, or vehicle) and a stirrer bar. The tubes were placed in the nonstirring wells of a multisample agitator (University of Nottingham, Nottingham, United Kingdom) operating at 37°C. When ADP was used the tubes were preincubated in the multisample agitator for 4 minutes before being transferred to the stirring wells; when adenosine was used, the tubes were transferred immediately to the stirring wells. In both cases, the tube contents were stirred for 1 minute, after which an aliquot (15 μL) of the sample was removed and mixed with fixative solution (30 μL) to provide a starting platelet count. After another minute, 20 μL of a solution of TRAP (20 μmol/L in PRP, 10 μmol/L in whole blood to reflect the difference in plasma volume) was added. Aliquots (15 μL) of the sample were removed at 1, 2, and 4 minutes and mixed with fixative solution (30 μL). The platelet count of the fixed samples was determined using the Sysmex KX-21 analyzer, an automated multitparamter blood cell counter. Platelet aggregation was calculated as the percentage loss of single platelets compared with the starting platelet count of PRP.

Measurement of Phosphorylation of VASP

Aliquots of PRP (final volume, 250 μL) were dispensed into prewarmed (37°C) polystyrene tubes (64×11 mm) containing the agent(s) under investigation or vehicle (10 μL). After 6 minutes, the reaction was stopped by the addition of ice-cold phosphate-buffered saline (1 mL), and the samples were placed on ice and transferred immediately to cold Eppendorf tubes. The samples were then centrifuged (11 600g, 10 seconds), the supernatant was discarded, and the resulting platelet pellet was lysed by vigorous resuspension in 100 μL of lysis buffer. Samples were then frozen and stored at −20°C before assay. The amounts of the phosphorylated form of VASP present in the lysates were measured by flow cytometry using a cytometric bead assay, which has been described previously. The assay was developed using Becton Dickinson Cytometric Bead Array products as described below. A specific activated protein C–fluorescent bead was chosen to prepare a stock of capture beads by conjugation of the beads to an appropriate capture antibody. Conjugation was performed according to instructions and reagents (Functional Bead Conjugation Buffer Set) with a monoclonal antibody to VASP (monoclonal antibody IE273 anti-VASP). The assay works on the principle that soluble VASP (phosphorylated and nonphosphorylated) in the platelet lysate is captured by the bead-bound antibody. Addition of a second fluorescent antibody (fluorescein isothiocyanate–conjugated antibody 5C6 anti-VASP pSer157) enables detection of the phosphorylated form of VASP on the capture bead by flow cytometry. Stock capture beads were diluted 1 in 50 using capture bead diluent (Cell Signaling Master Buffer Kit) before use. Lysed platelet samples were thawed and vortexed immediately before assay. Twenty-five microliters of sample was added to 25 μL of bead suspension in a fluorescence-activated cell sorting tube followed by the addition (7.5 μL) of fluorescein isothiocyanate–conjugated antibody 5C6 anti-VASP pSer157 (10 μg/mL) (Acris Antibodies). Samples were incubated in the dark at room temperature for 2 hours. Immediately before analysis, samples were resuspended in 150 μL of wash buffer (Cell Signaling Master Buffer Kit). Flow cytometry was performed on a Becton Dickinson LSRII flow cytometer using FACS Diva acquisition software using both the blue laser (488 nm) and red laser (17 mW power, excitation 633 nm) to perform the bead analyses. Capture beads were identified by their associated activated protein C.
fluorescence, and 300 bead events were collected. Phosphorylated VASP was detected as fluorescein isothiocyanate fluorescence expressed as median fluorescence.

Data Presentation
Data are presented as mean±SEM and were compared by repeated measures using SPSS 15 statistical software. Actual numbers of the different experiments performed are provided in the figure legends.

Results

Effects of ADP on TRAP-Induced Platelet Aggregation in PRP
We determined the effects of a range of concentrations of ADP on platelet aggregation in PRP induced by TRAP in the absence and presence of the P2Y12 receptor antagonists cangrelor, PAM, and ticagrelor. The PRP was incubated with the appropriate concentration of ADP for 6 minutes before the addition of TRAP. In the absence of a P2Y12 antagonist, the aggregation induced by TRAP was rapid, and preincubation with ADP did not inhibit this (results not shown). However, in the presence of a P2Y12 antagonist, TRAP caused a reversible aggregation response that was inhibited, in a concentration-dependent manner, by preincubation with ADP. The degree of inhibition brought about by ADP appeared to be the same regardless of the P2Y12 receptor antagonist used (Figure 1a, 1c, and 1e). At the same time, to establish the level of involvement of ADP-derived adenosine (as a result of ADP breakdown) in mediating the inhibition of the aggregation response to TRAP, these experiments were also performed in the presence of ADA (1.2 U/mL, a concentration that we had established as sufficient to remove the inhibitory effects of at least 10 μmol/L adenosine). In the presence of ADA, the inhibition brought about by preincubation with ADP was completely abolished (Figure 1b, 1d, and 1f).

Effects of ADP on TRAP-Induced Platelet Aggregation in Whole Blood
We then examined the effects of a range of concentrations of ADP on platelet aggregation in whole blood induced by TRAP in the absence and presence of P2Y12 antagonists and determined the effects of ADA, dipyridamole, and the combination of ADA and dipyridamole on this. The whole blood was incubated with the appropriate concentration of ADP for 6 minutes before the addition of TRAP. In the absence of cangrelor, PAM, or ticagrelor, the aggregation induced by TRAP was irreversible, and preincubation with ADP did not inhibit this (results not shown). In the presence of cangrelor, TRAP caused a reversible aggregation response that, in contrast to the results obtained in PRP, was not inhibited by preincubation with ADP (Figure 2a). Inhibition by ADP did occur when dipyridamole was also added to the blood (Figure 2b), and this inhibition was prevented by ADA (Figure 2c). Experiments were also performed using PAM (Figure 2d, 2e, and 2f) and ticagrelor (Figure 2g, 2h, and 2i) in place of cangrelor, and very similar results were obtained.

Effects of Adenosine on TRAP-Induced Platelet Aggregation in PRP
We investigated the ability of adenosine to inhibit TRAP-induced aggregation in PRP in the absence and presence of the P2Y12 antagonists cangrelor, PAM, and ticagrelor. In the absence of a P2Y12 antagonist, addition of TRAP to PRP brought about an irreversible aggregation response that was markedly inhibited by adenosine (Figure 3a). In the presence of P2Y12 antagonists, the aggregation response induced by TRAP was reversible. The degree of aggregation was the same irrespective of the P2Y12 antagonist used. Under these conditions, the addition of adenosine to the PRP clearly brought about a further inhibition of TRAP-induced aggregation (Figure 3b). Again, the degree of inhibition was the same irrespective of the P2Y12 antagonist used.

Effects of Adenosine on TRAP-Induced Platelet Aggregation in Whole Blood
We investigated the ability of adenosine with and without dipyridamole to inhibit TRAP-induced aggregation in whole blood, in the absence and presence of the P2Y12 antagonists. At the same time, we also looked at the ability of ADA to modify these effects. In the absence of a P2Y12 antagonist, addition of TRAP to whole blood brought about an irreversible aggregation response that was not modified by adenosine or dipyridamole alone, but there was a modest inhibition...
when adenosine and dipyridamole were used together (Figure 4a). This inhibition was prevented by ADA (Figure 4b). When the experiment was carried out in the presence of cangrelor, the aggregation response to TRAP became markedly reversible, and this was not modified by adenosine (Figure 4c). The addition of dipyridamole made the aggregation more reversible, whereas the addition of dipyridamole along with adenosine brought about almost complete inhibition of TRAP-induced aggregation. Once again, the inhibition was prevented by ADA (Figure 4d). Very similar results were obtained in the presence of PAM (Figure 4e and 4f) and ticagrelor (Figure 4g and 4h).

### Discussion

The focus of attention in this investigation was whether adenosine derived from ADP can contribute to inhibition of platelet aggregation, especially in the presence of a P2Y12 antagonist. ADP is broken down to AMP and then to adenosine in blood and plasma, and adenosine is able to inhibit platelet function through effects at A2A and A2B Gs-linked receptors to increase cAMP. In the presence of a P2Y12 antagonist, not only would the effects of ADP acting at P2Y12 Gi-linked receptors to reduce cAMP be prevented but the adenosine derived from it might be more able to bring about an inhibition of platelet function. There is already evidence that agonists that act at other Gs-linked receptors, including PGE1 and prostacyclin, are more able to inhibit platelet function in the presence of a P2Y12 antagonist.21,22 In the case of adenosine, however, the ability of adenosine to be rapidly taken up by red cells also needs to be considered in experiments performed in whole blood rather than in PRP.
Our initial experiments clearly demonstrated that ADP is indeed able to inhibit TRAP-induced platelet aggregation in the presence of a P2Y12 antagonist in PRP through conversion to adenosine. This was not the case in the absence of a P2Y12 antagonist. The involvement of adenosine was proven through complete abolition of the inhibition by ADA. The inhibition was very similar irrespective of which P2Y12 antagonist was used, cangrelor, PAM, or ticagrelor.

Such inhibition brought about by ADP in the presence of a P2Y12 antagonist was not seen when the platelet aggregation was measured in whole blood rather than PRP. However, the inhibition was restored when dipyridamole was added to the whole blood, and again the involvement of adenosine was proven using ADA. We and others have shown previously that dipyridamole very effectively inhibits the uptake of adenosine into red cells in whole blood and thereby leaves the adenosine available to interact with platelets. Once again, very similar results were obtained irrespective of the type of P2Y12 antagonist used.

To confirm the role of adenosine in platelet inhibition and the modulating effects of red cells present in whole blood, we performed some additional experiments in which we looked for platelet inhibition brought about directly by adenosine rather than by adenosine derived from ADP. As expected, we saw good inhibition of TRAP-induced platelet aggregation in PRP by adenosine. We also saw that the P2Y12 antagonists used alone converted an irreversible aggregation response to a reversible response, which we have demonstrated previously to be due to the ability of the P2Y12 antagonists to limit any contribution of secreted ADP to the aggregation obtained. We also saw that adenosine could add to the inhibition of platelet aggregation brought about by a P2Y12 antagonist. Also as expected, we saw no inhibition by adenosine in whole blood except when dipyridamole was also present, in line with adenosine uptake into red cells being an important contributor to the results obtained. ADA removed all the effects of the adenosine that was added. Once again, all the results were the same, irrespective of the P2Y12 antagonist that was used.

Studies of the effects of ADP on VASP phosphorylation in PRP provided clear evidence that adenosine derived from ADP is able to increase cAMP in platelets in the presence of any P2Y12 antagonist. This was not seen in the absence of a
P2Y₁₂ antagonist, where ADP interaction with P2Y₁₂ receptors clearly opposes the effects of the adenosine generated from the ADP acting at A₂A and A₂B receptors. It is interesting to note that many years ago in experiments performed using the P2Y₁₂ antagonist ARL66096, increased levels of cAMP were observed on adding ADP to PRP, and this was also prevented using ADA.³² In those experiments, the cAMP measurements were made in PRP that also contained a small amount of forskolin.

The question that arises from this investigation is the physiopathological significance of adenosine generation in vivo. Actually, ADP breakdown to adenosine is even more likely to occur in vivo given the abundant presence on endothelial cells of the enzymes CD39 and CD73 that contribute to ADP breakdown.¹⁶⁻¹⁹ However, the adenosine produced is very likely to be removed rapidly by red cells. One can speculate, however, that adenosine may indeed serve to modulate platelet function in areas where red cells are excluded. Such local effects might explain the observation that adenosine seems to be involved in the modulation of thrombus growth during experimental myocardial ischemia in dogs.¹⁵ Another consideration is the demonstration that hypoxia can lead to enhanced CD39 and CD73 activity,³³,³⁴ in addition to downregulation of the equilibrative nucleoside transporter²⁴,³⁵ and upregulation of the A₂B receptor,³³ all of which would be expected to increase the contribution of adenosine to platelet inhibition in cardiovascular disease. Perhaps a particularly interesting finding here is the ability of dipyridamole to promote the ability of adenosine derived from ADP to inhibit platelet function in the presence of a P2Y₁₂ antagonist in whole blood. We have already discussed the ability of dipyridamole to inhibit adenosine uptake into red cells, but in addition to its inhibitory effects on adenosine uptake, dipyridamole is also an inhibitor of cAMP phosphodiesterase.²⁷,²⁸ cAMP phosphodiesterase inhibitors inhibit platelet function by preventing the breakdown of cAMP, thus helping to maintain elevated intracellular levels of the nucleotide. Thus, in the presence of adenosine, this additional property could bring about even further inhibition of platelet aggregation. The ability of dipyridamole to enhance the antiplatelet effect of P2Y₁₂ antagonists will be of interest to those currently involved in clinical trials to assess the possible additional benefits of use of dipyridamole in combination with a P2Y₁₂ antagonist in some clinical scenarios, especially stroke prevention.³⁶⁻⁴⁰

In these experiments, we used 3 different P2Y₁₂ antagonists, cangrelor, PAM, and ticagrelor. We had expected that results obtained with ticagrelor may differ from those with cangrelor and PAM because of the reported ability of ticagrelor to inhibit uptake of adenosine into red cells, in the same way as dipyridamole does.²⁹ However, ticagrelor did not differ from the other 2 P2Y₁₂ antagonists in any way in the experiments that we performed and certainly did not reproduce the effects of a P2Y₁₂ antagonist in combination with dipyridamole. This suggests that any effect of ticagrelor on adenosine uptake is insufficient to amplify the antiplatelet effects of any adenosine generated in the presence of this particular P2Y₁₂ antagonist, at least under the conditions used in this study. Additional experiments would need to be performed to exclude possible potentiation by ticagrelor of adenosine-mediated effects in other cells and tissues in relation to cardioprotection and antiinflammatory responses.

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Disclosures
None.

References


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