In Vivo Blockade of Platelet ADP Receptor P2Y$_{12}$ Reduces Embolus and Thrombus Formation but Not Thrombus Stability

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Objective—ADP is a key platelet agonist in thromboembolism. One of the receptors involved in ADP-induced platelet activation is the P2Y$_{12}$ receptor, which is a target for antithrombotic drugs.

Methods and Results—Here, we present first evidence for a differential role of this receptor in thrombus and embolus formation in vivo. Anesthetized rabbits were treated with the selective P2Y$_{12}$ antagonists AR-C69931 MX (3 µg · kg$^{-1}$ · min$^{-1}$ IV) or clopidogrel (25 mg/kg orally). Efficacy of these treatments was monitored by aggregation and thrombin generation measurements in blood samples ex vivo. Mesenteric arterioles were mechanically injured; thrombus growth and subsequent embolus formation were visualized by real-time intravital microscopy. AR-C69931 MX and clopidogrel significantly ($P<0.05$) reduced the total duration of embolization (by 52% and 36%, respectively), and fewer and smaller emboli were produced. The size of the initial thrombus was significantly reduced ($P<0.005$), but its stability was unaffected; plug formation was still effective.

Conclusions—These findings demonstrate that ADP and its P2Y$_{12}$ receptor are involved in thrombus growth and especially in the formation of emboli on the downstream side of the initial thrombus. This may explain the beneficial effects of P2Y$_{12}$ receptor antagonists in secondary prevention of ischemic events in patients with arterial thrombosis. (Arterioscler Thromb Vasc Biol. 2003;23:518-523.)

Key Words: vessel wall injury ■ in vivo thromboembolism ■ platelet activation ■ adenosine diphosphate ■ antithrombotic drugs

Interactions between activated platelets and the vessel wall play a key role in normal hemostasis but also in vascular diseases such as arterial thrombosis. When platelets adhere to a damaged or diseased vessel wall, they become activated and recruit other platelets into an aggregate. Adherent platelets also expose a procoagulant surface on which thrombin can be formed; the resulting fibrin stabilizes the aggregate, and bleeding is stopped. However, this primary thrombus often remains highly thrombogenic, resulting in the subsequent production of ≥1 embolus. These secondary emboli may cause downstream vascular occlusions and lead to ischemia. Therefore, especially the embolization phase of the thromboembolic process is potentially hazardous. For instance, in patients with atherothrombotic disease, thrombi may form at the surface of an atherosclerotic plaque, and embolization may lead to myocardial or cerebral infarction or peripheral arterial ischemia.1

ADP is likely to play an important role in thromboembolism. Once released from dense granules on platelet activation, ADP amplifies the responses to other platelet agonists such as collagen and thrombin.2 ADP-induced platelet activation involves 2 receptors, P2Y$_{1}$ and P2Y$_{12}$, both of which contribute to full aggregation in vitro.3 The P2Y$_{1}$ receptor is coupled to the G$_{i}$ protein, causing mobilization of Ca$^{2+}$ ions to the cytosol, leading to shape change and initiation of aggregation.4,5 The recently cloned platelet-specific P2Y$_{12}$ receptor6,7 signals the inhibition of adenylate cyclase via G$_{i}$ and serves to complete and amplify the aggregation response to ADP.6,8 The P2Y$_{12}$-mediated signaling pathway involves the phosphoinositide 3-kinase–dependent activation of the platelet fibrinogen receptor $\alpha_{IIb}\beta_{3}$,9 and it has also been implicated in stabilization of platelet aggregates induced by thrombin receptor activation.10

Pharmacological agents inhibiting P2Y$_{12}$ receptors have found wide applications as antithrombotic drugs. For instance, the thienopyridine derivatives ticlopidine and clopidogrel, forming a metabolite with potent anti-P2Y$_{12}$ receptor activity,11 significantly reduce the risk of ischemic events in symptomatic atherothrombosis.12,13 Other novel P2Y$_{12}$ antagonists of the AR-C series14,15 also have demonstrated efficacy as antithrombotic drugs. However, not much is known about how these drugs interfere in the dynamic process of thrombus generation.
formation and subsequent embolization, because most of the in vivo studies on the role of the P2Y_{12} receptor in thrombotic processes provide information only on end-point parameters such as bleeding time or time to occlusion of a vessel.\textsuperscript{16} Therefore, it was the aim of the present study to investigate the mechanism underlying the antithrombotic effects of P2Y_{12} receptor inhibition in an established rabbit model of real-time thromboembolism in vivo\textsuperscript{17-20} by using the specific antagonists AR-C69931 MX (AR-CMX) and clopidogrel. For the first time, we provide evidence that blocking the P2Y_{12} receptor reduces thrombus formation and substantially diminishes embolization, without influencing the stability of the thrombus that sheds the emboli.

**Methods**

**Animal Preparation and Intravital Microscopy**

Experiments using laboratory animals were approved by the local ethics committee. These experiments were performed on New Zealand White rabbits (1.8 to 2.8 kg, n=33) of either sex as described previously.\textsuperscript{17-20} Rabbits were anesthetized by intramuscular injections of 40 mg ketamine hydrochloride (Nimatek, Eurovet) and 4 mg xylazine hydrochloride (Sedanum, Eurovet) per kilogram body weight; anesthesia was maintained by continuous intravenous infusion of ketamine (40 mg/kg per hour) and xylazine (5 mg/kg per hour), dissolved in lactertol (15 mL/H, Eurovet). Body temperature was kept at 37°C to 38°C. Arterial pressure and heart rate were continuously monitored by using an external pressure transducer (Uniflow, Baxter) connected to a catheter in the femoral artery. Rabbits were ventilated through a tracheal cannula with a mixture of nitrogen (74%), oxygen (25.5%), and carbon dioxide (0.5%) to control systemic arterial pH (7.46 ± 0.01 [mean ± SEM]), P_{O2} (83±3 mm Hg), and P_{CO2} (46±1 mm Hg). Arterial platelet counts and hematocrit values were assessed before the start of experimentation (Couler Counter, Coulter Electronics). Ear bleeding time was determined by making incisions (1 mm deep and 10 mm long) parallel to the long axis of the ear, avoiding macroscopically visible vessels. The incision site was carefully blotted with filter paper at 30-second intervals. Bleeding time was assessed from incision until the paper was no longer stained with blood.

Through a midline abdominal incision, a segment of the distal ileum was exteriorized. The mesentery was continuously superfused with buffered Tyrode’s solution (37°C, pH 7.35 to 7.40) and visualized with an intravital microscope (Leitz) with a long working distance objective (Leitz LL25, numerical aperture 0.35). Images were projected on a charge-coupled-device camera (Hamamatsu) and stored on videotape for offline analysis.

**Vessel Wall Puncture and Thromboembolic Reaction**

A thromboembolic reaction was induced in selected mesenteric arterioles (diameter 20 to 40 μm).\textsuperscript{17-20} After a stabilization period of 30 minutes, arterioles were mechanically injured by wall puncture using a glass micropipette with tip diameter of 6 to 8 μm. Puncture was considered successful if red blood cells were seen leaving the vessel, indicating that all vessel wall layers were damaged. In all vessels, bleeding stopped after a few seconds by the formation of a white platelet-rich thrombus. Circulating platelets continuously adhered to the downstream side of this stationary thrombus, and these newly formed aggregates embolized repeatedly. Embolization stopped after some time, while the thrombus remained unchanged at the site of injury during the observation period of 600 seconds per vessel. Arterioles were punctured up to 5 hours after the stabilization period.

The following parameters were quantified offline: duration of bleeding (microvessel bleeding time), occurrence of rebleedings, maximal thrombus height relative to local vessel diameter, total duration of embolization, number of emboli produced, size of individual emboli, and median embolus production time per vessel.

Emboli were counted when their short axis, perpendicular to the vessel wall, was >5 μm. Aggregates of smaller dimensions could not be distinguished from the background with sufficient accuracy.

**Administration of AR-CMX and Clopidogrel In Vivo**

The optimally effective dose of AR-CMX was determined ex vivo by whole-blood aggregometry in 4 rabbits. AR-CMX (Astra Zeneca) was infused via a catheter in an ear vein. Its dose was increased stepwise from 0 (saline=baseline) to 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μg/kg per minute. After each infusion step (20 minutes), 0.9 mL arterial blood was collected in 0.1 mL trisodium citrate (0.129 mol/L) via the arterial catheter. Whole-blood aggregation was induced by 20 μmol/L ADP and measured with an impedance aggregometer (Chronolog) at 37°C in the presence of H-Phe-Pro-Arg chloromethyl ketone (PPACK, 40 μmol/L) and CaCl_2 (16.6 mmol/L) to achieve physiological concentrations of free Ca^{2+}. Inhibition of the maximum level of aggregation by AR-CMX was determined relative to baseline level and calculated as follows: [baseline – AR-CMX/baseline]×100%. On the basis of these ex vivo measurements, an AR-CMX dose of 3 μg/kg per minute was selected for wall puncture experiments.

In a group of 8 rabbits, the compound was continuously infused via an ear vein (AR-CMX group). Continuous infusion was used because AR-CMX effects have been shown to rapidly recover on termination of infusion.\textsuperscript{17} Control rabbits (n=8) received continuous infusion of saline. In all rabbits, infusion (5 mL/h) was started at least 20 minutes before the first vessel was punctured. Rabbits were randomly assigned to the groups. The in vivo experiments and the offline analyses were performed blindly.

To compare the effects of AR-CMX with those of another well-known P2Y_{12} antagonist, an additional group of 3 rabbits was treated with an effective dose of clopidogrel (25 mg/kg).\textsuperscript{21} Clopidogrel was administered orally 2 hours before vessel puncture started. Arterial blood was collected in trisodium citrate (0.129 mol/L, 1/10 vol) before and 2 and 5 hours after clopidogrel administration. The maximal level of ADP-induced platelet aggregation (measured in platelet-rich plasma [PRP] as described below) was reduced by 62% after 2 hours and by 47% after 5 hours.

**Platelet Aggregation and Ca^{2+} Measurements**

Blood was collected in 1/10 vol trisodium citrate (0.129 mol/L) from a central ear artery of 4 rabbits to prepare PRP (2×10^8 platelets/mL). PRP was recalcified with 16.6 mmol/L CaCl_2 and PPACK (40 μmol/L) was added. ADP (20 μmol/L)-induced platelet aggregation was measured at 37°C by using a Chronolog optical aggregometer.

To measure Ca^{2+} responses, isolated rabbit platelets were loaded with fluo 3 as described.\textsuperscript{20} After immobilization on fibrinogen-coated coverslips, platelets were stimulated with 3 μmol/L ADP and 2 mmol/L CaCl_2, and fluorescence intensity changes in single platelets were measured.\textsuperscript{24} In addition, fluo 3-labeled platelets were injected into anesthetized rabbits (n=3), and fluorescence intensity changes in platelets participating in the thromboembolic process were measured as described previously.\textsuperscript{20}

**Thrombin Generation Assay**

Thrombin generation was measured in PRP by using the Thrombogram method.\textsuperscript{21} Blood was collected from 3 rabbits before (baseline) and after 30 minutes of infusion of AR-CMX at 3 μg/kg per minute. PRP (1.5×10^8 platelets/mL) was prepared and added to wells of a 96-well plate containing rabbit thromboplastin (1/30 000 vol Thromboplastin-S, Biopool International). Thrombin formation was started by adding CaCl_2 (16.6 mmol/L) and fluorescent thrombin substrate (417 μmol/L Z-Gly-Gly-Arg-AMC, Bachem) and continuously measured in time with a microtiter plate fluorometer (Fluo-
roskan Ascent, Labsystems) at 37°C. Control measurements were performed with platelet-poor plasma to ascertain that thrombin generation was platelet dependent.

**Statistical Analysis**

Data from in vivo experiments are presented as median values with (interquartile) ranges because of their nonsymmetrical distribution. Differences between the experimental groups were tested by the nonparametric Mann-Whitney U test. The Spearman rank correlation test was used to test correlations. All other data are presented as mean±SEM. Differences were tested by the paired t test.

**Results**

**Effects of P2Y12 Receptor Blockade on Rabbit Platelets**

To demonstrate whether P2Y12 mediates the activation of rabbit platelets, ADP-induced platelet aggregation in PRP was measured in vitro. AR-CMX reduced maximal aggregation by 44±5% at 1 μmol/L and by 66±6% at 100 μmol/L. Complete inhibition of aggregation was achieved only when a P2Y1 receptor antagonist was added as well, indicating that P2Y1 receptor activation accounted for the residual ADP-induced aggregation. To confirm that AR-CMX is specific for P2Y12, we investigated the effect of AR-CMX on Ca2+ responses in rabbit platelets. As expected, ADP-induced Ca2+ responses of single platelets in vitro were not influenced by preincubation with AR-CMX. In vivo, fluo 3-labeled platelets participated in the thromboembolic reaction, and individual labeled platelets were seen adhering to growing emboli. During AR-CMX infusion, the Ca2+ responses of these platelets were not different from control (data not shown). Hence, AR-CMX seems specific for the P2Y12 receptor in rabbit platelets.

The optimal AR-CMX dose for in vivo experiments was established ex vivo by use of whole blood aggregometry (Figure 1). Doses >1 μg/kg per minute significantly (P<0.05) decreased maximal aggregation: infusion of 1 μg/kg per minute reduced aggregation by 49±14%; infusion of 3 μg/kg per minute, by 71±6%; and infusion of 10 μg/kg per minute, by 80±5%. Because infusion of AR-CMX at 3 μg/kg per minute caused an almost maximal inhibition of aggregation (Figure 1), this dose was selected for subsequent in vivo experiments.

**Effect of P2Y12 Blockade on Thromboembolic Reaction In Vivo**

After the puncture of mesenteric arterioles (control, n=29; AR-CMX, n=36; and clopidogrel, n=19), bleeding and thrombus formation started immediately. Formation of the primary thrombus was completed within 1 to 2 seconds. AR-CMX reduced thrombus height by =20% (control median, 66%; AR-CMX, 54%; P<0.005; Figure 2). Microvessel bleeding time was not influenced (control, 4.9 seconds; interquartile range 2.3 to 7.7 seconds; AR-CMX, 2.9 seconds, interquartile range 1.3 to 10.7 seconds; P=0.57). After initial thrombus formation, rebleeding occurred in some vessels, but this frequency was similar in both groups (control, 24% of vessels; AR-CMX, 28% of vessels). Clopidogrel had similar effects: thrombus height was reduced to 37% (P<0.001, Figure 2) without significantly influencing microvessel bleeding time (3.1 seconds, interquartile range 1.9 to 5.8 seconds; P=0.29) or rebleeding frequency (37%, P=0.52). Thus, although the thrombus was smaller during P2Y12 blockade, its effectiveness to stop bleeding and prevent rebleeding was not influenced.

The formation of emboli on the downstream side of the thrombus was markedly reduced by AR-CMX (Figure 3). The total duration of embolization was decreased from 469 seconds in control to 228 seconds in AR-CMX arterioles (P<0.001, Figure 3a). In 11 of 29 control vessels, embolization continued for >600 seconds, whereas it stopped within 600 seconds in all but 1 AR-CMX arteriole. During the embolization period, on average, 14 visible emboli (short axis >5 μm) were produced in control arterioles, but only 8 were produced in AR-CMX vessels (P=0.001, Figure 3b). Moreover, AR-CMX decreased the size of these emboli: overall median embolus size per vessel was 10 to 15 μm in control arterioles and only 5 to 10 μm in AR-CMX vessels (P<0.01, Figure 3c). Because these observed emboli tended to get...
The effect of P2Y12 blockade on platelet-dependent thrombin formation was tested ex vivo. Infusion of AR-CMX at 3 \( \mu \text{g} / \text{kg} \) per minute reduced the peak value of thrombin generation by 25±7\% (\( P = 0.06 \), Figure 4). This parameter was not further reduced when PRP from blood collected after infusion was preincubated with AR-CMX (10 \( \mu \text{mol} / \text{L} \)) in vitro, indicating that infusion of AR-CMX at 3 \( \mu \text{g} / \text{kg} \) maximally reduced P2Y12-dependent thrombin formation. Preincubation of control blood with 10 \( \mu \text{mol} / \text{L} \) AR-CMX in vitro caused a reduction of thrombin generation of 42±11\%, which was not significantly different from the decrease caused by AR-CMX infusion (\( P = 0.24 \)). This suggests that the effective plasma concentration of AR-CMX during the in vivo experiments was in the micromolar range, near 10 \( \mu \text{mol} / \text{L} \).

Systemic Parameters and Fluid Dynamic Conditions

Ear bleeding time increased from 7.0 minutes (control) to 20.5 minutes (AR-CMX) during P2Y12 inhibition. Mean arterial blood pressure, heart rate, hematocrit, and platelet count were not different from control during AR-CMX or clopidogrel treatment. Overall medians and ranges were as follows: mean arterial pressure 69 (46 to 93) mm Hg, heart rate 148 (116 to 192) bpm, hematocrit 40\% (32\% to 53\%), and platelet count 520×10^3/L (268×10^3/L to 836×10^3/L). These values are within normal ranges for anesthetized rabbits.\(^{17,28}\)

In addition, P2Y12 blockade had no significant effect on local fluid dynamic parameters: arteriolar diameter (29 [19 to 46] \( \mu \text{m} \)), mean red blood cell velocity (1.8 [0.3 to 6.1] mm \( \cdot \) s\(^{-1} \)), and wall shear rate (943 [181 to 3605] s\(^{-1} \)) were not different between groups. These parameters were not significantly correlated with any of the thromboembolic parameters.

Discussion

This is the first study to show that (in damaged rabbit arterioles) blocking the platelet ADP receptor P2Y12 reduces initial thrombus formation and especially reduces the number and size of emboli shed by the initial thrombus, without influencing thrombus stability. The rate of embolization and the rate of adherence of individual platelets to growing
emboli are not decreased. The seemingly incompatible combination of an unchanged platelet adherence rate and the formation of smaller emboli indicates that even smaller and hence less harmful aggregates (<5 μm) are produced during P2Y12 blockade as well. This occurrence is probably due to relatively loose adhesion of platelets to the primary thrombus.

P2Y12 is the target of many antiplatelet drugs. The present in vivo findings provide insight into the way these drugs interfere in the dynamic process of thromboembolism. The present study shows that blockade of P2Y12 is especially effective during the embolization phase of a thromboembolic process. The duration of embolization was reduced by 40% to 50% with a correspondingly lower number of emboli; moreover, emboli were significantly smaller. If it is assumed that the process of platelet adhesion and aggregation that underlies embolization is similar in small and large vessels, these data may explain the effectiveness of P2Y12-inhibiting drugs in reducing the risk of ischemic events, such as myocardial or cerebral infarction or peripheral arterial ischemia in patients with atherothrombotic disease, because such ischemic events are considered to be the result of relatively large emboli shed from a thrombus.

If it is taken into account that P2Y12-mediated ADP effects are not influenced, the effects of P2Y12 blockade (by AR-CMX or clopidogrel) on embolization clearly indicate a prominent role for ADP during this phase of the thromboembolic process. Because emboli are of a loose nature and consist of weakly activated platelets compared with platelets in the thrombus, it is indeed likely that embolization involves platelet activation caused by relatively weak platelet agonists such as ADP. Activated platelets and vascular cells can also release thromboxane A2 (TXA2), which stimulates platelet aggregation. Previously, we have shown that in the same animal model, specific blockade of TXA2 receptors by sulotroban (BM 13,177) significantly reduces embolization in arterioles by >55%, without influencing thrombus size and stability. These effects of TXA2 receptor blockade on embolization (which can also be achieved by low doses of aspirin) are similar to those of P2Y12 blockade. The involvement of both TXA2 and ADP in embolization may explain why combined blockade of ADP and TXA2 pathways in patients is more effective in reducing ischemic events than is blocking the TXA2 pathway alone.

In addition to the pronounced inhibition of embolization, P2Y12 blockade resulted in a reduction of thrombus size. The biological relevance of this reduction can be questioned because rebleeding frequency was not influenced, indicating that the stability of the thrombus was unaffected. The reduction in thrombus size during P2Y12 blockade may be the consequence of a decrease in thrombin formation at the surface of procoagulant platelets. Because (in contrast to emboli) the primary thrombus is a stable aggregate with tightly packed, heavily shape-changed, and degranulated platelets that may be procoagulant, it is likely that thrombin is mainly formed in the thrombus. This is confirmed by pilot experiments in which a low molecular weight heparin was used: thrombus stability decreased, but embolization was unaffected (authors’ unpublished data, 2002). Thrombin can play a dual role in thrombus formation: (1) by activating platelets, it is (partly) responsible for thrombus size; and (2) by converting fibrinogen into fibrin, it stabilizes the thrombus. In the present study, we show that AR-CMX infusion indeed reduces the thrombin-generating capacity by ~25%, which may explain the decrease in thrombus size. Because thrombus stability is not influenced by P2Y12 blockade, the remaining thrombin is apparently able to produce sufficient fibrin. The reduction in thrombus size may also be due to inhibition of collagen-induced platelet activation, because in vitro studies have shown that collagen-induced platelet aggregation is impaired in platelets from P2Y12-deficient mice. In addition, in perfusion studies over collagen surfaces, thrombus size was found to be reduced when human platelets were deficient in P2Y12 or when this receptor was blocked by AR-CMX (in vitro) or clopidogrel (ex vivo).

The microvessel bleeding times presented in the present study are relatively short and unaffected by P2Y12 receptor blockade. Bleeding times as measured in mice and humans are substantially longer and prolonged when blocking the P2Y12 receptor. This discrepancy can likely be explained by the different types of vascular damage between the models used, being smaller in our model of vessel wall puncture and more severe in mice and humans, in whom larger vessels are transected, and surrounding tissue is opened as well. This is supported by our observation that P2Y12 blockade substantially increased bleeding time when there was more severe damage to the ear.

In summary, this is the first study in which the role of the ADP receptor P2Y12 is investigated in the thromboembolic process in vivo. Inhibition of this receptor especially reduces the production of potentially harmful emboli, without affecting the stability of the initial thrombus. Although our data were obtained in small rabbit arterioles, they may represent a general mechanism of action for P2Y12-inhibiting drugs in diseased human arteries. By shortening embolization duration and decreasing the size of emboli, the risk of downstream ischemia will be clearly reduced.

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