Blockade of the Platelet P2Y<sub>12</sub> Receptor by AR-C69931MX Sustains Coronary Artery Recanalization and Improves the Myocardial Tissue Perfusion in a Canine Thrombosis Model


Objective—Reperfusion therapy for myocardial infarction is limited by a significant reocclusion rate and less optimal myocardial tissue perfusion due to excessive platelet accumulation and recruitment at the sites of vascular injury. We assessed the influence of a selective P2Y<sub>12</sub>-receptor antagonist (AR-C69931MX), in conjunction with thrombolytic therapy, on the prevention of platelet aggregation and thrombus formation.

Methods and Results—A canine coronary electrolytic injury thrombosis model was used. Tissue-type plasminogen activator (t-PA; 1 mg/kg in phase I, 0.5 mg/kg in phase II in the AR-C69931MX group, and 1 mg/kg in the placebo group in phase I and II) was administered 30 minutes after thrombus formation; either saline or AR-C69931MX (4 μg · kg<sup>-1</sup> · min<sup>-1</sup>) was given to all animals intravenously 10 minutes before t-PA administration for a total of 2 hours. All animals received heparin (80 U/kg) as an intravenous bolus followed by a continuous infusion of 17 U · kg<sup>-1</sup> · h<sup>-1</sup>. Myocardial tissue perfusion was evaluated by use of the colored microsphere technique and real-time myocardial contrast echocardiography. The incidences of reocclusion and cyclic flow variation were significantly decreased in the AR-C69931MX group (P<0.05). Myocardial tissue flow with AR-C69931MX treatment improved significantly at 20 and 120 minutes after reflow, whereas tissue flow with placebo remained at a level similar to that during occlusion (P<0.05).

Conclusions—The adjunctive administration of AR-C69931MX blocked ADP-mediated platelet aggregation and recruitment and prevented platelet-mediated thrombosis, resulting in prolongation of reperfusion time and a decrease in reocclusion and cyclic flow variations. Importantly, myocardial tissue perfusion was significantly improved in the P2Y<sub>12</sub> antagonist group. (Arterioscler Thromb Vasc Biol. 2003;23:357-362.)

Key Words: P2Y<sub>12</sub> receptor ■ thrombosis ■ myocardial tissue perfusion ■ myocardial contrast echocardiography

Although much progress has been made in the treatment of ischemic heart disease during the past decades, acute ischemic complications due to excessive platelet accumulation, platelet recruitment, and thrombus formation remain a challenging therapeutic issue. Traditionally, successful reperfusion after acute myocardial infarction has been considered to be restored patency of epicardial coronary arteries. Increasing evidence indicates, however, that an open epicardial coronary artery may not represent “optimal reperfusion” and that adequate myocardial tissue perfusion is at least as important as sustained epicardial coronary patency. Lack of myocardial perfusion at the tissue level, despite restoration of normal antegrade flow in the epicardial infarct-related artery, predicts poor recovery of left ventricular function in acute myocardial infarction. ADP-induced platelet activation plays a pivotal role in thrombus formation and thus, inhibition of this pathway holds promise as a means of significantly reducing clot formation and improving myocardial tissue reperfusion during acute ischemic states. There are 2 subtypes of ADP receptor on platelets: P2Y<sub>1</sub> and P2Y<sub>12</sub> receptor. P2Y<sub>1</sub> is found in other tissues, whereas the P2Y<sub>12</sub> receptor, previously referred to as P<sub>2T</sub>, is found only on platelets, megakaryocytes, and astroglial cells of the brain.2-6

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The thienopyridines, including ticlopidine and clopidogrel, are platelet ADP receptor antagonists that act at the level of P2Y<sub>12</sub> receptor.7,8 However, their effects on platelets are slow and irreversible,9 and they show only moderate inhibition of ADP-induced platelet aggregation at the current therapeutic doses.10 ATP is the natural antagonist of ADP-induced platelet aggregation through competitive binding of the plate-
let ADP P2Y12 receptor. The ATP analogue AR-C69931MX, a novel, selective, and specific P2Y12 receptor antagonist, was recently synthesized. Initial animal studies suggested that this analogue inhibited arterial thrombosis with less effect on bleeding time compared with platelet glycoprotein IIb/IIIa receptor antagonists. Recently, AR-C69931MX has been shown to be effective in preventing the development of thrombosis in a canine model of electrolytic carotid arterial injury. In this study, we evaluated the effect of AR-C69931MX, in conjunction with thrombolytic therapy, on reperfusion and the prevention of reocclusion in a canine coronary thrombosis model. Furthermore, the influence of AR-C69931MX on myocardial microvascular reperfusion and tissue perfusion was studied by myocardial contrast echocardiography (MCE).

Methods

Surgical Preparation

A canine coronary electrolytic injury thrombosis model was used as described previously with hound dogs (20 to 22 kg) of either sex. In brief, after induction of anesthesia with intravenous sodium pentobarbital (25 mg/kg) and intubation, a left thoracotomy was performed through the fifth intercostal space. A 2-cm segment of left circumflex coronary artery (LCX) was isolated, and a Doppler ultrasonic flow probe (Crystal Biotech) was placed around the vessel for the measurement of coronary blood flow (CBF). CBF was monitored and recorded on a digital data-acquisition system (Gould Instrument System, Inc). Thrombosis was induced with the electrolytic injury technique. The endothelium of the LCX was manually injured by gently rubbing the artery distal to the flow probe. A coronary electrode, consisting of silver-coated copper wire with a 26-gauge needle tip, was then inserted into the isolated and injured segment of the LCX, ensuring its contact with the intraluminal surface of the vessel. Visual inspection for proper position of the electrode in the artery during the experiment was performed, and visual evidence of vessel wall injury surrounding the electrode was assessed at the end of experiment. Distal to the flow probe and the electrode, a vascular occluder was placed around the vessel and then adjusted to induce 80% stenosis. Thrombosis was initiated by delivery of a 100-μA continuous anodal current to the tip of the coronary electrode, and formation of a fully occlusive coronary thrombus was determined by zero CBF. The occluder was gradually removed, and electrical stimulation was then suspended. Throughout the procedure, the electrocardiogram was continuously monitored to determine heart rate and S-T segment elevation. Blood gas analysis was performed to maintain the pH and arterial blood gases within physiological range. All experiments conformed to the position of the American Heart Association on research animal use and care and were conducted with the approval of the Animal Research Committee of the Cleveland Clinic Foundation.

Experimental Protocol and Drug Administration

All animals received aspirin (325 mg) orally on the day of the procedure. After the occluder was removed and electrical stimulation was turned off, 30 minutes were allowed to elapse to confirm the stability of thrombosis before administration of tissue-type plasminogen activator (t-PA; Figure 1). Twenty minutes after thrombus formation (10 minutes before t-PA was given), the dogs were randomized to receive either saline or AR-C69931MX (4 μg · kg−1 · min−1) intravenously for a total of 2 hours. Simultaneously with administration of t-PA, heparin was given as an 80 U/kg IV bolus followed by a continuous infusion of 17 U · kg−1 · h−1 for the subsequent duration of the experiment.

Two phases were included in this study. In phase I, t-PA (1 mg/kg) was administered IV to all animals (placebo group = 10, AR-C69931MX group = 10) for 20 minutes. In phase II, the dogs from the placebo group (n = 10) were given 1 mg/kg t-PA, whereas only 0.5 mg/kg t-PA was administered to the animals in the AR-C69931MX group (n = 10). The purpose of this second phase was to investigate whether prevention of reocclusion and cyclic flow variation (CFV) could still be achieved by a lower dose of t-PA combined with AR-C69931MX adjunctive therapy. In phase II, myocardial tissue blood flow (TBF) was evaluated with a standard fluorescent color microsphere technique and MCE at baseline, during occlusion, and at the end of the experiments.

Figure 1. Schematic diagram of experimental protocol. Thrombus formation is induced by passage of 100-μA continuous anodal current through the coronary electrode. Twenty minutes after thrombus formation, dogs received either AR-C69931MX (4 μg · kg−1 · min−1) or saline. Ten minutes afterward, t-PA was given to all animals for a 20-minute period (phase I, 1 mg/kg; phase II, 0.5 mg/kg). Colored microspheres were injected directly into the left atrium before thrombus formation, as control, during the occlusion, and at the end of adjunctive treatment administration. MCE was performed before thrombus formation, during occlusion, 20 minutes after t-PA infusion, and at the end of adjunctive treatment administration. All animals from both phases received heparin (80 U/kg as a bolus, 17 U · kg−1 · min−1 IV).

Thrombolysis was defined as restoration of CBF to at least 30% of the baseline value, occurring at any time after the onset of drug infusion. Reocclusion was defined as occurrence of zero CBF after successful thrombolysis. The animals were observed for a 2-hour period after the occurrence of thrombolysis and evidence of reocclusion. If reocclusion did not occur, the maximal duration of reperfusion was considered to be 120 minutes. In those animals with intermittent CBF restoration due to CFVs, the total duration of reperfusion was calculated as the length of time during which blood flow was greater than zero during the 2-hour period after onset of reperfusion. Two hours after the onset of thrombolysis, animals with persistent zero blood flow as well as those with CFVs were considered to have reocclusion of the coronary artery. Xylocaine was administered to control ventricular arrhythmias when it was necessary.

Measurement of Myocardial TBF

In phase II, myocardial TBF was measured with a standard fluorescent color microsphere technique on a blinded basis. Three different fluorescent color microspheres (Triton Technology Inc) were injected directly into the left atrium at baseline, during occlusion, and at the end of the experiment. Reference blood samples were simultaneously withdrawn from the carotid artery at a predetermined rate of 5.8 mL/min by a calibrated withdrawal pump (Harvard Apparatus). Tissue samples from the epicardial and endocardial regions of the area at risk and nonischemic area were taken. After tissue digestion, the microsphere content was spectrophotometrically measured (spectrofluorometer FP-777). The value of TBF in the area at risk and nonischemic area was calculated as the average of corresponding epicardial and endocardial flows.

Myocardial Contrast Echocardiography

In phase II, real-time harmonic MCE was performed at baseline, during occlusion, 20 minutes after t-PA infusion, and at the end of
Table 1. Thrombolytic Effects of tPA with Heparin, AR-C69931MX

<table>
<thead>
<tr>
<th>Phase</th>
<th>Placebo (n=10)</th>
<th>AR-C69931MX (n=10)</th>
<th>Placebo (n=10)</th>
<th>AR-C69931MX (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reperfusion rate, %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Time to reflow, min</td>
<td>24.7±7.9</td>
<td>20.9±9.1</td>
<td>21.8±6.4</td>
<td>21.8±5.8</td>
</tr>
<tr>
<td>Reflow duration time, min</td>
<td>75.0±39.9</td>
<td>120±0*</td>
<td>86.1±45.2</td>
<td>120±0*</td>
</tr>
<tr>
<td>Cyclic flow variation rate, %</td>
<td>50</td>
<td>0*</td>
<td>20</td>
<td>0*</td>
</tr>
<tr>
<td>Reocclusion rate, %</td>
<td>60</td>
<td>0*</td>
<td>40</td>
<td>0*</td>
</tr>
</tbody>
</table>

*P<0.05 compared with placebo group.

Statistical Analysis
Statistical analysis was performed with use of SPSS software (version 7.0 for Windows, SPSS Inc). Data are presented as mean±SD. Continuous variables were compared by unpaired t tests. Perfusion data were analyzed with a repeated-measures ANOVA with a post hoc Tukey’s honestly significant difference test. A value of P≤0.05 was considered to be statistically significant.

Results
Effects of Adjunctive AR-C69931MX on Thrombolysis and Acute Reocclusion
The comparative thrombolytic effects in the 2 groups are shown in Table 1. Baseline CBF was similar in the 2 groups in both phases. The mean time required for occlusive thrombus formation was also similar in the 2 groups (37.6±14.3 minutes in the AR-C69931MX groups vs 41.7±9.8 minutes in the placebo groups, P=NS). No acceleration in thrombolysis or increased reperfusion rate was observed in AR-C69931MX groups compared with placebo groups. However, there were significant differences in the incidence of reocclusion and CFV between the 2 groups. In AR-C69931MX groups, there was no reocclusion or CFV, but reocclusion occurred in 6 of 10 animals (phase I) and in 4 of 10 animals (phase II), and CFV occurred in 5 of 10 animals (phase I) and in 2 of 10 animals (phase II) in the placebo groups.

Myocardial TBF
Myocardial TBF in the risk area as measured by colored microspheres was similar at baseline in the 2 groups (0.93±0.65 mL · min⁻¹ · g⁻¹ with placebo and 0.90±0.44 mL · min⁻¹ · g⁻¹ with AR-C69931MX group, P=NS). During coronary occlusion, TBF within the risk area was reduced to 0.30±0.19 mL · min⁻¹ · g⁻¹ in the placebo group and to 0.32±0.36 mL · min⁻¹ · g⁻¹ in the AR-C69931MX group (P=NS). By 120 minutes after initiation of the study drug, TBF with AR-C69931MX improved to 0.69±0.41 mL · min⁻¹ · g⁻¹, whereas TBF with placebo remained at 0.35±0.20 mL · min⁻¹ · g⁻¹ (P<0.05) (Figure 2).

Myocardial Contrast Echocardiography
There was no difference in myocardial TBF at baseline and during occlusion between the 2 groups. However, the AR-C69931 MX group showed better microvascular perfusion than the placebo group (Figures 3 and 4) at 20 minutes (20.31±13.71 dB/s vs 5.42±4.43 dB/s; P=0.01) and 120 minutes (29.95±17.89 dB/s vs 8.56±6.16 dB/s; P<0.001) after thrombolysis. Moreover, the correlation between MCE
findings and triphenyltetrazolium chloride staining for assessment of the area at risk and infarct size was excellent ($r=0.87$, $P<0.0001$)

**Platelet Aggregation**

The results of platelet-rich plasma platelet aggregation in response to ADP alone and ADP plus epinephrine are summarized in Table 2. There was no influence of t-PA alone on platelet aggregation. However, adjunctive administration of AR-C69931MX significantly decreased platelet aggregation by $>98\%$ (ADP alone) and $79\%$ (ADP plus epinephrine).

**Coagulation Study and Bleeding Time**

After administration of t-PA, prothrombin time and activated partial thromboplastin time values were increased in all animals as expected, without any difference between the 2 groups. By the end of adjunctive therapy, there was no significant difference in prothrombin time and activated partial thromboplastin time between the 2 groups. Bleeding time was significantly prolonged (1.8- to 2.8-fold) owing to AR-C69931MX administration compared with the placebo group (end of t-PA, 3.68±1.64 minutes in the placebo group vs 9.85±0.049 minutes in the AR-C69931MX group, $P<0.001$; end of adjunctive therapy, 2.48±0.61 minutes in the placebo group vs 4.36±1.14 minutes in the AR-C69931MX group, $P<0.001$).

**Infarct Size**

In both phases, infarct size was significantly reduced by $\approx50\%$ in the AR-C69931MX group when expressed either as percentage of the area at risk or as absolute infarct size (Table 3).

**Discussion**

ADP is a key mediator of platelet aggregation and thrombosis, particularly under high-shear flow conditions characteristic of stenosed atherosclerotic arteries. In this study, we demonstrated that inhibition of ADP-mediated platelet aggregation and recruitment by the platelet ADP receptor-P2Y$_{12}$ receptor antagonist AR-C69931MX significantly prolonged reperfusion time and abolished reocclusion and CFVs, even with half of the dosage of t-PA in the same canine thrombosis/fibrinolysis model. These beneficial effects were achieved with only a modest increase in bleeding time in comparison with placebo. Most importantly, this study shows that combination therapy with a thrombolytic agent and antagonism of platelet aggregation not only maintains patency of epicardial coronary arteries but also improves myocardial microvascular perfusion and myocardial salvage.

Adenine nucleotides affect a number of cellular events. Activation of platelets by ADP is a receptor-mediated process involving 2 subtypes of the P2 receptor family: P2Y$_4$ and P2Y$_{12}$. The latter subtype (also historically referred to as P$_{2Y}$, P2Y$_{AC}$, P$_{2Y_{12}}$, P$_{2Y_{ADP}}$) is found on platelets, megakaryocytes, and astroglial cells of the brain. It has been demonstrated that P2Y$_{12}$ receptor activation is important for the sustained platelet-aggregation response to ADP and for a significant component of the platelet response to other agonists.$^{20,21}$ The importance of this receptor pathway in arterial thrombosis is also suggested by the positive clinical findings with the thienopyridines, ticlopidine and clopidogrel,$^{22,23}$ which produce partial inhibition of P2Y$_{12}$-mediated platelet activation after hepatic generation of an active metabolite.$^{24}$ Humbert and colleagues$^{25}$ further studied ADP-dependent platelet aggregation by using platelets from a patient lacking...
the P2Y₁₂ receptor and normal platelets in the presence of clopidogrel. They found that ADP-induced aggregates of the patient’s platelets and clopidogrel-treated normal platelets are composed of a small number of “loosely packed” platelets with fewer than normal contact points. Recently, Nurden et al. and Remijn et al. confirmed these findings and demonstrated that P2Y₁₂ receptor antagonist. In contrast to clopidogrel, its abnormal, fragile platelet aggregates.

AR-C69931MX was similarly impaired. The thrombi were smaller and morphologically abnormal in blood from the patient and control blood in the presence of AR-C69931MX. Thus, it is unclear whether the antithrombotic effect of AR-C69931MX is due to inhibition of platelet aggregation or to the formation of abnormal, fragile platelet aggregates.

AR-C69931MX is a novel, highly potent, and selective P2Y₁₂ receptor antagonist. In contrast to clopidogrel, its antiplatelet activity is not dependent on metabolic conversion, and in both healthy volunteers and patients, potent and complete inhibition of ADP-induced platelet aggregation has been observed ex vivo during intravenous infusion. The pharmacokinetic profile of AR-C69931MX is characterized by rapid attainment of a steady-state plasma concentration and a rapid offset of effect on cessation of infusion. Huang and colleagues have shown that AR-C69931MX displayed a rapid onset and offset of action with the ability to prevent occlusive arterial thrombus formation. This rapidly reversible characteristic may make this agent well suited for inhibiting platelet function under appropriate clinical conditions while reducing the risk of bleeding complications.

An important finding of this study is that restoration and maintenance of myocardial microvascular flow and tissue perfusion were achieved with combination therapy of a thrombolytic agent and AR-C69931MX, as evidenced by MCE and myocardial TBF measurement. In the placebo group, the compromised myocardial tissue perfusion was not limited to the animals with CFV or reocclusion; a similar finding was observed in the animals without CFV or reocclusion, indicating that the difference in myocardial tissue perfusion was due to improvement of myocardium perfusion rather than a higher patency rate in the AR-C69931MX group. Increasing evidence suggests that although the patency of the infarct-related artery may have been reestablished, disordered microvascular function and inadequate myocardial tissue perfusion are often present after reperfusion therapy for acute myocardial infarction. Thus, optimal reperfusion should not only sustain epicardial patency but also restore microvascular flow and myocardial tissue perfusion. The potential explanation for this finding is that potent antplatelet therapy may relieve microvascular obstructions caused by platelet-thrombin microemboli.

In summary, AR-C69931MX is a potent and selective inhibitor of ADP-induced platelet aggregation through its P2Y₁₂ receptor antagonistic property. The administration of AR-C69931MX in the canine coronary thrombosis model blocked ADP-induced platelet aggregation and recruitment and prevented platelet-mediated thrombosis. It prolongs reperfusion time, abolishes reocclusion and CFVs, and restores myocardial tissue perfusion with a modest increase in bleeding time. This agent may be a useful adjunct during reperfusion therapy for acute myocardial infarction in humans.

### Acknowledgment

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### Table 3. Myocardial Area at Risk and Effect of AR-C69931MX on Infarct Size

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=10)</th>
<th>AR-C69931MX (n=10)</th>
<th>Placebo (n=10)</th>
<th>AR-C69931MX (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area at risk, cm²</td>
<td>42.37±3.89</td>
<td>43.26±2.64</td>
<td>55.08±4.17</td>
<td>54.09±3.85</td>
</tr>
<tr>
<td>Infarct size, cm²</td>
<td>9.34±4.37</td>
<td>4.70±4.66*</td>
<td>17.79±7.50</td>
<td>9.87±5.89*</td>
</tr>
<tr>
<td>Percentage of infarct size at risk area, %</td>
<td>19.37±9.3</td>
<td>9.19±9.0*</td>
<td>32.47±13.93</td>
<td>18.33±11.52*</td>
</tr>
</tbody>
</table>

*P<0.05 compared with placebo group.
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