Antagonism of P2Y12 or GPIIb/IIIa receptors reduces platelet-mediated myocardial injury after ischaemia and reperfusion in isolated rat hearts

José A. Barrabés; Javier Inserte; Maribel Mirabet; Adoración Quiroga; Víctor Hernando; Jaume Figueras; David García-Dorado
Servicio de Cardiología, Hospital Universitari Vall d’Hebron, Barcelona, Spain

Summary
Platelets activated during experimental acute myocardial infarction (AMI) contribute to myocardial injury. This study aimed to investigate whether platelets from patients with AMI increase myocardial injury after ischaemia and reperfusion in isolated rat hearts and the modification of this effect by the P2Y12 receptor antagonist cangrelor and the glycoprotein IIb/IIIa receptor blocker abciximab. Isolated rat hearts were subjected to 40 minutes of global ischaemia and 60 minutes of reperfusion. Hearts (four simultaneous experiments per patient) were infused with platelets from nine AMI patients (seven thrombolysed, all on aspirin), untreated or incubated with 10 μM cangrelor or 5 μg/ml abciximab. Control experiments were performed using platelets from healthy volunteers and platelet-poor plasma. P-selectin expression on isolated platelets was higher in AMI patients than in controls and was not modified by the treatments. Control platelets or platelet-poor plasma did mild or no harm. In contrast, platelets from AMI patients significantly augmented myocardial injury, as demonstrated by worse left ventricular (LV) developed pressure, higher maximal LV end-diastolic pressure and coronary resistance, and greater lactate dehydrogenase release and infarct size. Both cangrelor and abciximab greatly attenuated these effects. In conclusion, activated platelets from AMI patients increase myocardial injury after ischaemia and reperfusion, and cangrelor and abciximab attenuate this effect. The results support the notion that early antiplatelet treatment might reduce infarct size by direct effects on reperfused myocardium in these patients.

Keywords
Ischaemia, microcirculation, platelets, reperfusion, antithrombotic agents

Introduction
Platelets are central to the pathophysiology of acute myocardial infarction (AMI) not only because of their role in thrombus formation and growth at the culprit coronary lesion but also through their contribution to myocardial injury at the tissue level (1–3). Platelets interact with reperfused endothelium and accumulate in the microvasculature, where they can enhance myocardial damage by hampering blood flow or by releasing toxic substances (1–10). On the other hand, circulating platelets are activated by ischaemia and reperfusion (6, 9, 11–15) and their activation status correlates with myocardial damage both in patients (12, 14, 16) and laboratory animals (6, 9, 15).

Some antiplatelet drugs blocking the P2Y12 receptor or the glycoprotein (GP) IIb/IIIa receptor have been shown to also antagonize other molecules involved in microvascular platelet deposition after reperfusion such as the vitronectin receptor or the Mac-1 integrin (17–20). Although these drugs have favorably influenced myocardial perfusion after experimental coronary thrombosis or in AMI patients and have improved the outcome of the latter (21–28), whether they increase myocardial salvage by direct effects on the microcirculation is unclear (24, 29–31).

Accordingly, we aimed to assess the effects of platelets obtained from patients with AMI on myocardial damage after transient ischaemia in isolated rat hearts and the modification of these effects by P2Y12 receptor and GPIIb/IIIa receptor blockade. To achieve this, we used cangrelor, a P2Y12 receptor antagonist with a rapid effect after intravenous administration that also inhibits platelet P-selectin expression and platelet-leukocyte co-operation (20, 26), and abciximab, the chimeric monoclonal antibody fragment that blocks the GPIIb/IIIa receptor as well as other molecules involved in platelet adhesion to reperfused endothelium (17–19). Platelets from healthy volunteers were used as controls.

Materials and methods

Blood sampling
The protocol obtained institutional approval and experiments were performed in accordance with the Declaration of Helsinki. After obtaining informed consent, 20–30 ml of blood was with-

Correspondence to:
Dr. David García-Dorado
Servicio de Cardiología
Hospital Universitari Vall d’Hebron
Pg. Vall d’Hebron 119–129
08035 Barcelona, Spain
Tel.: +34 93 489 4038, Fax: +34 93 489 4032
E-mail: dgordaro@vhebron.net

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drawn from a peripheral vein in seven untreated, healthy volunteers and in nine patients with an AMI of <24 hours (h) evolution (15.0 ± 2.4 h) and not having received any antplatelet drugs other than aspirin. Blood was collected in syringes containing 1/10 vol. of 3.8% citrated solution.

**Platelet aggregometry**

Ten milliliters of blood were used to perform platelet aggregometry. Platelet-rich plasma (PRP) was obtained by centrifugation for 12 minutes (min) at 150 g and platelet-poor plasma (PPP) by centrifugation for 15 min at 1,800 g at room temperature. Platelet concentration was adjusted to 300,000/μl by adding PPP if necessary. Optical aggregometry was performed in a four-channel 570 SV aggregometer (Chrono-log Corporation, Havertown, PA, USA) at 1,000 rpm and 37ºC. PRP samples were stirred for 5 min with either saline, 10 μM cangrelor (kindly provided by The Medicines Company, Parsippany, NJ, USA) or 5 μg/ml abciximab (ReoPro® , Centocor, Leiden, The Netherlands) and aggregation was induced by adding 20 μM ADP and measured as the maximal increase in light transmittance as a percentage of that observed with PPP. Drug concentrations were selected after assessing in previous dose-ranging experiments that they induced a near-maximal anti-aggregatory effect on platelets from healthy volunteers (data not shown).

**Platelet isolation for infusion into isolated rat hearts and analysis of P-selectin expression**

Twenty milliliters of blood were immediately treated with prostacyclin (2 μg/ml, final concentration) and centrifuged at 200 g for 15 min at room temperature. The upper 2/3 of the supernatant (PRP) was collected and centrifuged at 900 g for 10 min. The supernatant was removed and the platelet pellet was washed at 700 g for 10 min with modified HEPES-Tyrode’s buffer (129 mM NaCl, 2.8 mM KCl, 0.8 mM KH₂PO₄, 5 mM glucose, 10 mM HEPES, 0.02% BSA, pH 7.4) containing 0.3 μg/ml prostacyclin (6, 7). Platelets were re-suspended in modified HEPES-Tyrode’s buffer, counted with a Coulter STKS cell counter and the concentration adjusted to 45 x 10⁶ platelets/ml. The platelet suspension was divided into three aliquots which were treated for 5 min at 37ºC with either saline, 10 μM cangrelor or 5 μg/ml abciximab before infusion into isolated rat hearts at a final concentration of 40 x 10⁶ platelets/ml.

Platelet P-selectin expression was assessed by flow cytometry. Briefly, aliquots of platelet suspensions were incubated for 30 min at room temperature with a FITC-conjugated anti-P-selectin monoclonal antibody (clone MOPC-21, BD Biosciences Pharmingen, San Jose, CA, USA), washed with PBS-0.5% FBS and re-suspended in the same buffer. Negative controls were performed using an irrelevant isotype-matched FITC-conjugated antibody (clone AK-4, BD Biosciences Pharmingen). The platelet population was analysed at a low flow rate in a FACScalibur flow cytometer (Becton Dickinson Inc., San Jose, CA, USA) calibrated weekly for fluorescence and light scatter using fluorescent beads. Platelets were identified on the basis of forward and sideward scatter parameters in the logarithmic mode. For each sample 20,000 platelets were collected. Data were analysed with CELLQuest™ software (Becton Dickinson) and results were expressed as percentage of specific antibody-positive platelets, defined as those with a fluorescence intensity exceeding that of 99% of negative control platelets. The absence of microaggregates, which could cause by themselves myocardial injury (1), in the platelet suspensions was assessed by flow cytometry.

**Isolated rat heart preparation and experimental protocol**

Male Sprague-Dawley rats weighing 300–350 g (n = 50) were anesthetised with intraperitoneal injection of 150 mg/kg sodium pen-

![Figure 1: Experimental design. AMI, acute myocardial infarction; CAN, cangrelor; ABC, abciximab.](image)
tobarbital. Hearts were removed, mounted into a non-recirculating Langendorff apparatus and perfused at 10 ml/min with modified Krebs-Henseleit bicarbonate buffer equilibrated with 95% O₂ and 5% CO₂ as previously described (6, 7).

The experimental design is illustrated in Figure 1. After 30 min of equilibration, hearts were subjected to 40 min of global ischaemia followed by 60 min of reperfusion. Four simultaneous experiments per patient were performed: one heart received no additional intervention and the remaining three hearts were infused during the last 5 min of equilibration with platelets (22.5 x 10⁶/min), either untreated or incubated with cangrelor or abciximab. The cells were infused into the coronary flow at a constant rate of 0.5 ml/min through a sidearm of the Langendorff apparatus just proximal to the heart inflow cannula (6, 7). Experiments with platelets from healthy volunteers along with their own controls were performed on separate days. To exclude effects mediated by agents in the infused plasma, four additional experiments were performed in which rat hearts (four per experiment) were infused with either buffer, washed platelets obtained from patients with AMI of <24 h evolution, or an equivalent volume of PPP.

**Results**

**Clinical characteristics of patients**

Clinical characteristics of patients are summarised in Table 1. The seven patients presenting with ST-elevation had received teneclase with significant improvement of the electrocardiographic changes. None of the patients had heart failure or other complications with a single exception that presented with an episode of primary ventricular fibrillation shortly after the onset of symptoms that was successfully defibrillated. All patients were on aspirin and full-dose enoxaparin at the time of blood sampling.

**Platelet aggregometry and P-selectin expression**

In patients, blood platelet count was 311 ± 48 x 10⁶/μl and ADP-induced platelet aggregation averaged 48 ± 6% and was reduced to 9 ± 1% by cangrelor (p < 0.001) and to 5 ± 1% by abciximab (p < 0.001). The relative inhibition of aggregation was 78 ± 4% for cangrelor and 90 ± 2% for abciximab (p = not significant [NS]).

P-selectin expression in isolated platelets from healthy volunteers averaged 18 ± 1%. In AMI patients, P-selectin expression was 28 ± 4% in untreated platelets (p = 0.03 with respect to controls).

**Statistical analysis**

Statistical analysis was performed using SPSS software. Values are expressed as individual data or means ± standard error of the mean (SEM). One-way analysis of variance was used to assess differences among the groups, followed by the less significant difference test for individual comparisons when the overall result was significant. Values of p < 0.05 were considered significant.
26 ± 4% in platelets incubated with cangrelor and 31 ± 5% in platelets treated with abciximab (p = NS for the comparison between these three groups).

LV pressure and coronary perfusion pressure in isolated rat hearts

An example of LV pressure tracings is provided in Figure 2. At the end of the equilibration period, LVEDP averaged 7 ± 1 mmHg, LV developed pressure 101 ± 4 mmHg, and coronary perfusion pressure 62 ± 2 mmHg. No-flow ischaemia resulted in cessation of contractile activity and in a steep increase in LVEDP that reached a peak of 71 ± 6 mmHg 15 ± 1 min after the onset of the ischaemic period. No differences among the groups were observed during equilibration or ischaemic periods.

In buffer-perfused hearts, reperfusion induced a further increase in LVEDP of 95 ± 5 mmHg at 4.2 ± 0.2 min after reflow, and a functional recovery of variable magnitude was observed during this period. Whereas infusion of control platelets only mildly and non-significantly affected these functional parameters, hearts receiving platelets from AMI patients showed earlier and greater hypercontracture (Fig. 3A and B), worsened functional recovery (Fig. 3C) and higher increase in coronary resistance (Fig. 3D). These deleterious effects were attenuated to a variable extent both by cangrelor and abciximab (Fig. 3).

Quantification of cell death

In buffer-perfused hearts, total LDH release during the reperfusion period was 296 ± 19 U/gdw (grams dry weight) and infarct size averaged 38 ± 2% of ventricular mass. LDH release and infarct size were not significantly influenced by infusion of control platelets but were clearly increased in hearts infused with platelets from AMI patients, and this increase was significantly blunted both by cangrelor and abciximab (Fig. 4). In the control experiments, platelet infusion also increased LDH release during the first 60 min after reperfusion (593 ± 66 U/gdw vs. 368 ± 20 U/gdw in buffer-perfused hearts, p = 0.004), but PPP caused no significant harm (421 ± 39 U/gdw, p = NS vs. buffer, p = 0.021 vs. platelets).

Discussion

This study investigated the effects of platelets obtained from healthy controls or from patients with AMI on myocardial injury secondary to ischaemia and reperfusion in isolated rat hearts and the modification of these effects by P2Y12 or GP IIb/IIIa receptor blockade. Control platelets did mild or no harm but platelets from patients with AMI exacerbated ischaemia-reperfusion injury, as shown by worse functional recovery, increased hypercontracture and coronary resistance, and greater myocardial necrosis. These effects were significantly attenuated by platelet incubation with the

![Figure 2: Example of functional monitoring.](image-url)

LVP, left ventricular pressure; AMI, acute myocardial infarction.
Figure 3: Functional data. Timing (A) and magnitude (B) of hypercontracture (maximal left ventricular [LV] end-diastolic pressure [LVEDP]) at early reperfusion, and LV developed pressure (C) and perfusion pressure (D) at 60 minutes of reperfusion in the five groups. Data are mean ± SEM. CP, control platelets; IP, infarction platelets; CAN, cangrelor; ABC, abciximab. N = 9 hearts per group except for buffer (N = 16) and CP (N = 7). *P < 0.05 vs. IP group.

Figure 4: Data on myocardial damage. Total lactic dehydrogenase (LDH) release (A) and infarct size (B) in the five groups. Data are mean ± SEM. CP, control platelets; IP, infarction platelets; CAN, cangrelor; ABC, abciximab. N = 9 hearts per group except for buffer (N = 16) and CP (N = 7). *P < 0.05 vs. IP group.
P2Y₁₂ receptor antagonist cangrelor or the GP IIb/IIIa receptor blocker abciximab.

There is increasing evidence that platelets contribute to myocardial injury after reperfusion by direct effects at the tissue level (1, 2). Platelets adhere to reperfused endothelium by several mechanisms including P-selectin-ligand interactions (32), GPIIb/IIIa binding to fibrinogen (33) or via the vitronectin receptor (34). Platelet infusion into isolated hearts of different animal species subjected to transient ischaemia has worsened blood flow or contractile function and increased myocardial necrosis (6, 7, 9, 35, 36). In addition, animals with genetically impaired platelet function display an increased tolerance to ischaemia (8, 10). The deleterious effect of platelets on reperfused myocardium appears highly dependent on their activation status, since the infusion of activated platelets obtained from animals with ongoing AMI significantly increased myocardial injury whereas platelets from sham-operated animals caused no harm in isolated rat or mice hearts (6, 9). The disparity in activation status between platelets isolated from healthy volunteers and from AMI patients observed in the present study and their differential effects on reperfused hearts are consistent with previous observations using animal platelets (6, 7, 9) and highlight the potentially toxic effect of circulating activated platelets despite standard antithrombotic treatment in this clinical setting.

As aspirin lacks anti-adhesive properties, its favourable influence on outcome in AMI patients is largely attributed to the prevention of coronary re-occlusion (37) and is probably unrelated to any microvascular effects (5, 38, 39). The ability of P2Y₁₂ or GPIIb/IIIa receptor blockers to reduce the expression of molecules involved in platelet adhesion to reperfused endothelium (17–20) increases the likelihood they have a protective role beyond stabilization of the culprit lesion. However, this issue remains under dispute despite the large amount of clinical data available. This is in part because of the difficulties in dissecting the effects of these drugs on the downstream myocardium from their facilitation of reperfusion or prevention of re-occlusion. These latter phenomena have a strong influence on myocardial damage and microvascular integrity.

In a sub-study of the CLARITY-TIMI 28 (Clopidogrel as Adjunctive Reperfusion Therapy-Thrombolysis in Myocardial Infarction 28) trial, clopidogrel failed to increase the proportion of patients with ST resolution, an established surrogate for myocardial reperfusion, 90 min after thrombolysis in 2,431 patients with ST-elevation AMI (31). Since platelet adhesion to reperfused endothelium and platelet-mediated myocardial damage occur very early after reperfusion, as shown in the present study and others (6, 7, 9, 32, 33), the slow onset of action of clopidogrel may underlie this apparent lack of effect on the microcirculation. In line with this interpretation, clopidogrel pretreatment has recently been associated with better angiographic myocardial perfusion at the end of primary percutaneous intervention for ST-elevation AMI (25). Cangrelor is a potent P2Y₁₂ receptor antagonist with a very rapid onset of action after intravenous administration and which also inhibits ADP-induced P-selectin expression and platelet-leukocyte co-operation (20). Cangrelor has recently been shown to have no superiority compared to an oral loading dose of clopidogrel prior to percutaneous intervention in an effort to reduce early ischaemic complications in patients with a broad spectrum of coronary artery disease (40). However, experimental and clinical data have suggested that this compound could facilitate myocardial reperfusion as an adjunct to thrombolysis in AMI (26, 41). In our study, cangrelor significantly protected the heart from platelet-mediated injury, further supporting the value of early P2Y₁₂ receptor blockade in the acute treatment of AMI.

GP IIb/IIIa inhibitors are associated with improved tissue perfusion after reperfusion therapy in AMI (21, 22, 42). However, this has been translated into reduced mortality only in patients treated with primary percutaneous intervention, probably because of an excess of bleeding in those receiving thrombolytics (24). Overall, observational and experimental studies suggest that early GP IIb/ IIIa inhibition improves myocardial perfusion and may be associated with improved survival as compared with peri-procedural administration, although data in this respect are heterogeneous (24, 43). The results of our study, where abciximab exerted a strong protective effect against myocardial damage induced by activated platelets, support the early use of these agents in AMI.

We had previously observed no benefit of small-molecule inhibitors against reperfusion injury in isolated rat hearts perfused with activated platelets (6) or in pigs subjected to transient coronary occlusion (29). The superior performance of abciximab is consistent with its singular effects on other mechanisms of platelet adhesion including the vitronectin receptor or Mac-1 integrin (17–19) and concurs with the finding that simultaneous inhibition of GPIIb/IIIa and vitronectin receptors during reperfusion is more protective than GP IIb/IIIa receptor blockade alone (30).

Several methodological considerations and potential limitations should be discussed. First, patients and control subjects differed with regard to the presence of co-morbidities that could affect platelet function, although this should not have interfered with the observed effects of cangrelor and abciximab. A significant confounding effect of differences in plasma composition, including those related to the presence of aspirin or other drugs, was excluded by control experiments. Second, we cannot determine whether the greater myocardial injury induced by platelets from
AMI patients was due to their higher P-selectin expression as compared to control platelets. However, previous studies have shown that antagonising P-selectin function, a major mechanism of platelet adhesion to reperfused endothelium, protects against platelet-mediated damage (7, 9, 35). Third, we did not include patients on thienopyridines to avoid interference of this treatment in the assessment of the effects of cangrelor. This resulted in the predominant inclusion of patients receiving thrombolysis, although the results can probably be extrapolated to all patients with reperfused AMI irrespective of the mode of reperfusion. Fourth, we did not assess the effect of the combination of cangrelor and abciximab because of the technical impossibility to study more than four hearts simultaneously. Given the strong beneficial effect observed with each of these drugs in isolation, it seems unlikely that this combination would provide additional protection in our model. Finally, the lack of differences in platelet P-selectin expression between untreated platelets and platelets incubated with cangrelor or abciximab is at variance with previous reports that abciximab increases (44, 45) and cangrelor reduces (20) platelet activation and P-selectin expression. The fact that incubation and flow cytometry were performed after platelet isolation, which induced significant α-degranulation, may help explain this discrepancy. In addition, all patients had received aspirin, which may prevent some of these effects (44). The translation of these results into a clinical improvement in the complex setting of ST-elevation AMI is uncertain. However, working with such controlled experimental conditions allows a better understanding of the mechanisms of reperfusion injury and the modes of action of these drugs. This may help interpret the results of clinical studies and identify the patient subgroups in which a maximal benefit could be expected.

In conclusion, the present results provide additional evidence of the role of activated platelets in myocardial injury after ischaemia and reperfusion and support the notion that early antiplatelet treatment targeting platelet adhesion may increase myocardial salvage by direct effects on the microcirculation in patients with ST-elevation AMI.

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References


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