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Platelet P2Y₁₂ Blockers Confer Direct Postconditioning-like Protection in Reperfused Rabbit Hearts

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Abstract

Background—Blockade of platelet activation during primary percutaneous intervention for acute myocardial infarction is standard care to minimize stent thrombosis. To determine whether antiplatelet agents offer any direct cardioprotective effect, we tested whether they could modify infarction in a rabbit model of ischemia/reperfusion caused by reversible ligation of a coronary artery.

Methods and Results—The P2Y₁₂ (adenosine diphosphate) receptor blocker cangrelor administered shortly before reperfusion in rabbits undergoing 30-minute regional ischemia/3-hour reperfusion reduced infarction from 38% of ischemic zone in control hearts to only 19%. Protection was dose dependent and correlated with the degree of inhibition of platelet aggregation. Protection was comparable to that seen with ischemic postconditioning (IPOC). Cangrelor protection, but not its inhibition of platelet aggregation, was abolished by the same signaling inhibitors that block protection from IPOC suggesting protection resulted from protective signaling rather than anticoagulation. As with IPOC, protection was lost when cangrelor administration was delayed until 10 minutes after reperfusion and no added protection was seen when cangrelor and IPOC were combined. These findings suggest both IPOC and cangrelor may protect by the same mechanism. No protection was seen when cangrelor was used in crystalloid-perfused isolated hearts indicating some component in whole blood is required for protection. Clopidogrel had a very slow onset of action requiring 2 days of treatment before platelets were inhibited, and only then the hearts were protected. Signaling inhibitors given just prior to reperfusion blocked clopidogrel's protection. Neither aspirin nor heparin was protective.

Conclusions—Clopidogrel and cangrelor protected rabbit hearts against infarction. The mechanism appears to involve signal transduction during reperfusion rather than inhibition of intravascular coagulation. We hypothesize that both drugs protect by activating IPOC's protective signaling to prevent reperfusion injury. If true, patients receiving P2Y₁₂ inhibitors before percutaneous intervention may already be postconditioned thus explaining failure of recent clinical trials of postconditioning drugs

Keywords

cangrelor; clopidogrel; myocardial infarction; platelet; postconditioning

INTRODUCTION

Myocardial infarctions are caused by coronary plaque rupture and intraluminal thrombosis. The platelet is instrumental in the genesis of this flow-stopping thrombus through a choreographed series of steps initiated by tethering of platelets to endothelial cells followed by their binding to exposed collagen through glycoprotein (GP) VI and integrin $\alpha 2\beta 1$ receptors. Those events trigger a complex signaling cascade leading to cross-linking of platelets and aggregation. This process is amplified by activation of platelet surface receptors for P2Y₁₂ and thromboxane (TBX) A₂.

Pharmacologic approaches have been used to combat platelet aggregation in patients with coronary thrombosis and/or thrombogenic intracoronary stents. Frequently used agents include aspirin that blocks cyclooxygenase and generation of TBX. The thienopyridines clopidogrel and prasugrel or the adenosine triphosphate analogs cangrelor and ticagrelor interfere with adenosine diphosphate (ADP) binding to the P2Y₁₂ receptor. Others such as the antibody fragment abciximab block fibrinogen binding to GPIIb/IIIa.

The exact role of platelets in myocardial infarction is not clear. When infused into hearts subjected to ischemia/reperfusion, platelets activated by ischemia or pharmacologic agents increase myocardial necrosis,¹ and nonactivated platelets infused into isolated hearts subjected to ischemia/reperfusion increase the extent of ischemia² and adversely affect postreperfusion hemodynamics.³ Conversely, one study has suggested platelets have cardioprotective properties.⁴ In mice with either Gq,⁵ GPVI,^{6,7} or P-selectin⁸ deficiencies, platelet aggregation is minimized and infarct size is reduced following ischemia/reperfusion.

Platelet aggregation in the microcirculation of reperfused myocardium could obviously extend infarction. Accordingly, most studies have found that GPIIb/IIIa antagonists diminish infarct size and sequelae of ischemia/reperfusion in animals^{9–15} and man.^{16,17} However, there have also been contradictory reports.^{1,13,15,18} A high loading dose of clopidogrel was shown to limit infarct size in patients with acute myocardial infarction undergoing primary angioplasty.¹⁹ In that and similar studies the effect on infarction has generally been attributed to the prevention of intravascular coagulation.

Recently, several postconditioning agents that performed well in preclinical studies yielded disappointing results in clinical trials in patients with acute myocardial infarction.^{20,21} One possible explanation is that one or more of the many drugs that these patients receive might unknowingly be a postconditioning agent leading to the speculation that these patients were actually already protected before the test agent was administered. A likely culprit would be antiplatelet agents. We, therefore, asked whether such an agent might have a direct anti-infarct effect in our rabbit model in which coronary flow is interrupted with a ligature rather than a thrombus. We tested the oral P2Y₁₂ blocker, clopidogrel, that must not only be absorbed but also be metabolized from a prodrug to an active form. Although platelet inhibition can be seen within an hour of ingestion in humans,²² 2 days of oral treatment were required until platelets were inhibited in our rabbits. We, therefore, turned to cangrelor, a second P2Y₁₂ inhibitor, that can be given intravenously and acts immediately. We found that both of these anti-aggregatory agents are indeed highly protective against infarction, and our experiments suggest that they may do so by preventing a reperfusion injury by activating the well-known conditioning pathway rather than by preventing intravascular coagulation. If they directly condition the heart, then that could explain why adding a conditioning intervention would offer no additional protection.

Methods

Surgical Preparation

All protocols were approved by the Institutional Animal Care and Use Committee of the University of South Alabama College of Medicine and conformed to published guidelines.²³ New Zealand White rabbits of either gender were anesthetized with 30 mg/kg intravenous sodium pentobarbital. Additional intravenous boluses of 5 mg/kg were administered approximately every 30 minutes to maintain a surgical plane of anesthesia. Animals were mechanically ventilated with 100% O₂. After a left thoracotomy, the heart was exposed and a snare was placed around a prominent branch of the left coronary artery. Arterial blood pressure was measured via a femoral artery catheter and continuously recorded. After equilibration, arterial blood was withdrawn for measurement of platelet aggregometry from the femoral artery catheter. Arterial blood for platelet aggregometry was withdrawn 1 minute prior to the end of the 30-minute coronary occlusion. In some rabbits, arterial blood for platelet aggregometry was also obtained after 1 hour of reperfusion.

For two protocols, hearts were excised after placing the snare around the coronary artery and perfused with oxygenated Krebs buffer on a Langendorff apparatus as previously detailed.²⁴ Left ventricular developed pressure and heart rate were continuously recorded. The snare was tightened to create regional ischemia. Coronary flow was measured by timing collection of effluent dripping from the heart.

Infarct Area and Risk Zone

As previously described,²⁵ the heart was removed and after reoccluding the coronary artery 2 to 9 μ m green fluorescent microspheres (Microgenics Corp, Fremont, California) in saline were retrogradely infused into the heart through the aortic root. The risk area was thus myocardium without fluorescent microspheres. The heart was quickly frozen and cut into ~3 mm slices perpendicular to its long axis. Slices were incubated for 8 to 10 minutes in 1% triphenyltetrazolium chloride (GFS Chemicals, Powell, Ohio) at 37°C and then put into 10% formalin for tissue preservation and enhancement of color contrast between living (stained) and infarcted (unstained) tissue. Risk zone regions identified under ultraviolet light and infarcted regions under white light were traced on plastic overlays. Areas were measured by planimetry and volumes were calculated by multiplying areas by slice thickness. Infarct size is presented as percentage of risk zone volume.

Platelet Aggregometry

Aggregation was determined by measuring impedance with a whole blood aggregometer (Chrono-log Corp, Havertown, Pennsylvania). A 0.5 mL of saline and a 0.5 mL of heparinized arterial blood were combined in a plastic cuvette and continuously stirred. Except for the aspirin studies aggregation was initiated by addition of either 5 or 10 μ L of 1 mmol/L ADP to produce final concentrations of 5 and 10 μ mol/L, respectively. When aspirin was administered, arachidonic acid was added instead of ADP to make a final concentration of 0.1 mmol/L. If there was no aggregation, additional aliquots were added to create a final concentration of 0.35 and then 0.85 mmol/L.

Protocols

Twenty open-chest groups were studied. All rabbits experienced 30-minute coronary occlusion/3-hour reperfusion. Control experiments received no treatment and were performed periodically throughout the study to ensure that all animals responded similarly to ischemia/reperfusion. Hence the control group is larger than all others.

Clopidogrel

Three groups were used to investigate the effect of oral treatment with clopidogrel, a prodrug that must be activated in the liver by cytochrome P450 enzymes. Pilot studies revealed negligible platelet inhibition or protection 24 hours after oral dosing with 37.5 mg of pulverized clopidogrel in molasses (~15 mg/kg), but oral dosing for 2 days did inhibit platelet reactivity. Hence, we used the latter dosing schedule. Two of the clopidogrel-treated groups were co-treated with either wortmannin or MRS1754 injected intravenously 5 minutes before reperfusion to test for dependence on signal transduction (see below for doses).

Cangrelor

Cangrelor was used as the antiplatelet agent in 14 groups. Unless otherwise stated, cangrelor-treated rabbits received a standard dose of 60 µg/kg intravenous (iv) bolus 10 minutes before reperfusion and an infusion of 6 µg/kg per minute was continued throughout reperfusion. To obtain dose–response data, rabbits were treated with either one-fourth (15 µg/kg bolus and 1.5 µg/kg per min infusion) or one-eighth (7.5 µg/kg bolus and 0.75 µg/kg per min infusion) of the original dose of cangrelor. Infusion was for 3 hours. In 2 groups, we studied the effect of shortening the infusion by stopping it at either 1 or 2 hours. In 1 group, we tested whether the drug had to be present in the first minutes of reperfusion by delaying cangrelor's administration until 10 minutes after the onset of reperfusion.

Two groups were used to investigate interaction between ischemic postconditioning (IPOC) and cangrelor. The IPOC was effected with 4 cycles of 30-second reperfusion/30-second reocclusion immediately after release of the 30-minute coronary occlusion. In a second group, cangrelor infusion was combined with IPOC.

To test whether signal transduction was involved in cangrelor's protection, we co-administered cangrelor and a signaling antagonist. The antagonist was injected intravenously 15 minutes before reperfusion (5 minutes before cangrelor) at a concentration known to block cardioprotective effects of ischemic or pharmacological postconditioning²⁶: wortmannin (60 µg/kg) and LY294002 (0.3 mg/kg), PI3 kinase/Akt antagonists; PD98059 (0.3 mg/kg), antagonist of MEK1/2, and, therefore, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2); 5-hydroxydecanoic acid (5 mg/kg), putative blocker of mitochondrial KATP channels despite occasional contrary report²⁷; 8-sulfophenyltheophylline (7.5 mg/kg), a nonselective antagonist of adenosine receptors; MRS1754 (9.5 µg/kg), a selective antagonist of adenosine A_{2B} receptors; and 2-mercaptopropionylglycine (25 mg/kg bolus followed by 1 mg/kg per min for 45 minutes), a scavenger of reactive oxygen species and known to block redox signaling.

Aspirin and heparin

We investigated 2 other anticoagulant drugs, aspirin and heparin. Rabbits were treated with iv acetylsalicylic acid (aspirin), 3 mg/kg per hour, started 30 minutes before occlusion and continuing for 90 minutes or intravenous boluses of 500 U heparin 10 minutes before reperfusion and again 60 minutes later.

Isolated heart investigations

Finally, in 2 groups an isolated rabbit heart model of myocardial infarction was used to determine whether blood components are needed for cangrelor's protection. Krebs buffer-perfused hearts were subjected to 30-minute regional ischemia/2-hour reperfusion. In 1 group, hearts were switched to buffer containing 200 nmol/L of cangrelor 5 minutes prior to reperfusion and this enhanced buffer was continued until the end of reperfusion.

Statistics

Baseline hemodynamics for all groups were compared by one-way analysis of variance (ANOVA). Hemodynamic changes within any given group were evaluated by ANOVA for repeated measures. Infarct sizes were analyzed by one-way ANOVA. For all statistical analyses, post hoc testing was done with the Student-Newman-Keuls test. For statistical analysis of infarct sizes, ANOVA was performed on the groupings depicted in Figures 1C, 2, 3, 4, and 5C. The platelet aggregation curves were analyzed by determining slopes and areas beneath the curves. Only the area data are presented since the slope data yielded similar conclusions. All curves were truncated at 5 minutes. Curves following administration of a drug were always compared to the baseline curve obtained before drug treatment and paired t tests used to determine statistical significance of differences in areas beneath the curves. A P value of <.05 was considered to be significant.

RESULTS

In vivo infarct size experiments were completed in 130 rabbits. Blood pressure and heart rate data are presented in Table 1. There were no differences among the groups at baseline except for a higher diastolic pressure in hearts with delayed cangrelor treatment. Neither platelet antagonist affected hemodynamics. Blood pressure decreased during coronary occlusion and tended to recover following reperfusion in all groups. Infarct sizes are summarized in Table 2. Risk zone volume did not differ among groups except in rabbits treated with cangrelor plus PD98059 where it was larger. The effect of the various treatments on platelet aggregation is shown in Table 3.

Clopidogrel

Despite a very large oral dose of clopidogrel no effect on platelet aggregation or infarct size (data not shown) was seen 24 hours later. Dosing for a second day, however, did inhibit the aggregation response to ADP (Figure 1A) and also limited the infarct size (Figure 1C). The protection against infarction was blocked by either wortmannin or MRS1754 indicating that signal transduction was involved in the protection. Neither of these inhibitors interfered with the inhibition of aggregation which suggests that prevention of coagulation was not the cause of the protection. The inhibitors were given just prior to reperfusion. Therefore, they must have blocked protective signaling that occurred during the reperfusion period.

Cangrelor

Because clopidogrel's slow onset of effect hampered our studies, we looked for another P2Y₁₂ blocker that could be given parenterally. We, therefore, tested cangrelor at the dose recommended by the manufacturer (60 µg/kg iv bolus followed by 6 µg/kg per min for the duration of reperfusion). The infusion is required because cangrelor (in humans) has only a 9-min half-life.²⁹ Cangrelor treatment was started 10 minutes before reperfusion. Cangrelor was also very effective in blocking platelet aggregation in rabbits as shown in Figure 1B. Figure 1C reveals that cangrelor could limit infarct size by an amount similar to that of clopidogrel. In all rabbits, this dose of cangrelor decreased aggregation by more than 94% (Table 3).

Figure 2 reveals the dose and schedule requirements for cangrelor. Cangrelor infusion for only 70 minutes was not long enough to protect. However, a 130-minute infusion did protect despite absence of drug infusion during the final hour of reperfusion. When administration of our standard cangrelor dose was delayed until 10 minutes following the onset of reperfusion, protection was lost indicating that the first minutes of reperfusion are critical for the protection. When the dose of cangrelor was decreased to one-fourth of our standard dose aggregation was blocked by 72% and protection was still evident. Further, diminution to

one-eighth of the original dose caused only 50% blockade of aggregation and protection was lost (Figure 2). Thus cangrelor protection was correlated with platelet aggregation.

The finding that cangrelor had to be present in the first minutes of reperfusion suggested a postconditioning mechanism. As previously reported,²⁸ rabbits subjected to IPOC were protected and infarct size was similar to that seen with cangrelor (Figure 2). When IPOC was combined with cangrelor treatment, no further reduction in infarct size was seen (Figure 2), which would be expected if both used the same mechanism. To further test our postconditioning hypothesis, we used blockers of signal transduction that have been shown to block IPOC's protection²⁶ in our model. When signaling elements including adenosine A_{2B} receptors, ERK, Akt, redox signaling, or mitochondrial KATP channels were individually blocked, cangrelor protection was aborted (Figure 3). Because none of the inhibitors diminished cangrelor's ability to block aggregation, we concluded that signal transduction rather than inhibition of aggregation was responsible for cangrelor's protection.

Isolated Heart Investigations

We studied 15 isolated hearts, 9 control and 6 treated with cangrelor. Hemodynamics, including coronary flow, were not different in the 2 groups. Although isolated rabbit hearts can be both pre- and postconditioned,^{24,30} cangrelor had no effect on infarct size (Figure 4). These experiments indicate that cangrelor's protection was not caused by some nonspecific effect of the cangrelor molecule. Rather, cangrelor must interact with some blood-borne element, presumably platelets, to produce its protective effect.

Aspirin and Heparin

Low-dose arachidonic acid activated platelets (Figure 5A). Aspirin blocked platelet aggregation from low dose arachidonic acid but not from ADP (Figure 5B). Aspirin did not significantly affect infarction (Figure 5C). The range of infarct sizes in this group was uncharacteristically large, suggesting that aspirin might have had a small protective effect in some hearts. Heparin tended to increase the infarct size (Figure 5C).

DISCUSSION

Blockade of P2Y₁₂ receptors with 2 chemically and biologically distinct antagonists was found to be cardioprotective in rabbit hearts. Both P2Y₁₂ inhibitors decreased infarction by approximately 50% which is similar to that seen with IPOC. Although aspirin also inhibited platelet aggregation, it did so only in response to arachidonic acid. Aspirin did not significantly reduce the infarct size although there was a trend toward smaller infarcts. Heparin was also not protective.

Based on the 4 following points, we propose that cangrelor and clopidogrel may act as postconditioning mimetics. First, their protective effect is abrogated by antagonists of the same signaling steps required by ischemic pre- and postconditioning. The kinase and receptor "fingerprint" of postconditioning's and platelet-blockade's protection was found to be identical. Second, none of these inhibitors interferes with cangrelor's ability to block platelet aggregation, again suggesting that signal transduction rather than any effect on aggregation per se causes the protection. Third, the combination of IPOC and cangrelor produced no additional effect than that already seen with cangrelor or IPOC individually. This observation would be expected if they protected by the same mechanism. Fourth, postconditioning is thought to protect by inhibiting permeability transition pore formation in the mitochondria in the first minutes of reperfusion.²⁶ Therefore, at least in the rabbit model, IPOC must be instituted in the first minutes of reperfusion to protect.²⁸ Delaying

administration of cangrelor until 10 minutes after reperfusion causes complete loss of protection indicating that its protection is also conferred in the first minutes of reperfusion.

Oral clopidogrel had a very slow onset of action in our rabbits despite receiving a dose 50% larger than the recommended loading dose for humans. It is unknown whether this is related to a difference in bioavailability or impaired hepatic conversion to the active form. As a result, it was not possible to give clopidogrel just before reperfusion to see if it is truly a postconditioning mimetic. However, like cangrelor its protection relied on signaling and correlated with the ability to block platelet aggregation suggesting that it might also use the conditioning mechanism. Since the drug would have been present at reperfusion in these hearts, the fact that it had to be given prior to the onset of ischemia does not eliminate the possibility that it actually protected at reperfusion. Administration of either wortmannin or MRS1754 just before reperfusion blocked protection in the clopidogrel-treated rabbits and this indicates that protection did occur during reperfusion. And, because neither signaling antagonist blocked clopidogrel's antiplatelet effect and restored platelet reactivity in those animals, signaling rather than anticoagulation must have caused the protection. Protection against infarction from a reperfusion injury through the conditioning mechanism may be a class effect and hence a characteristic of all P2Y₁₂ blockers.

Current guidelines recommend the use of antagonists of platelet aggregation in patients undergoing primary angioplasty. The mechanism by which these agents improve outcomes has been assumed to be related to their ability to maintain microcirculation patency, but in light of the present findings this may not be the case. We propose that these agents may trigger the same cardioprotective signaling pathway used by pre- and postconditioning.²⁶

Over the past decade, there have been sporadic reports regarding the efficacy of platelet antiaggregatory compounds on infarct size in experimental animals. In many of these studies^{12,15} infarction was induced by a thrombus which was then dissolved by tissue plasminogen activator. In that case, platelet inhibition would have hastened thrombolysis thus shortening the ischemic period, and hence obscuring any direct protective effect of the drug on infarction. However, several studies used ligation to occlude the coronary artery and thus the duration of the ischemic period was precisely controlled. Kingma et al¹⁰ observed infarct sparing after treatment with a GPIIb/IIIa antagonist in dogs. Because there was no effect of the agent on either collateral flow during ischemia or on myocardial flow during reperfusion, the authors also proposed a direct protective effect on the heart muscle by some unknown mechanism. Several other studies reported benefit in canine hearts, but unfortunately collateral flow was not accounted for in the data analysis thus complicating the infarct size analysis.^{31,32} On the other hand, Kunichika et al¹⁴ and Sakuma and colleagues¹¹ did account for collateral flow and observed that tirofiban administered prior to reperfusion in dogs decreased the infarct size. Thus, these 3 studies corroborate our findings although all used GPIIb/IIIa inhibiting agents rather than a P2Y₁₂ inhibitor. We could find only 1 comparable study with a P2Y₁₂ blocker, but it was in pigs. Pig myocardium, like that of rabbits, is collateral poor so the variability in collateral flow does not need to be included in the analysis. Three days of treatment with clopidogrel plus aspirin in the pigs did not decrease the infarct size.¹³ The failure to protect may reflect a species difference or efficacy may be lost either with prolonged administration of clopidogrel or when it is combined with aspirin. Our testing of clopidogrel was made on the first day that aggregation was abolished and we did not test its combination with aspirin.

Eptifibatide, the one GPIIb/IIIa inhibitor to which we had access, did not inhibit rabbit platelet aggregation (data not shown), so we do not know whether inhibition of GPIIb/IIIa would be protective in our model. Although the 3 studies mentioned above reported protection from a GPIIb/IIIa agent, we found a fourth study that did not.¹⁸ Except for the

suggestion by Kingma et al,¹⁰ authors of the other 2 positive studies attributed the reduced infarction to better perfusion.

We do not know why blocking platelet aggregation just prior to reperfusion might postcondition the heart. Pre- and postconditioning are initiated by occupation of G protein-coupled receptors (GPCRs) which trigger the protective signaling.²⁶ It is difficult to postulate how blocking aggregation releases a triggering substance(s) that turns on the pathway since disaggregation is the normal status for blood in healthy hearts. The mystery of cangrelor's and clopidogrel's cardioprotection might be explained if these drugs nonselectively activated preconditioning-related GPCRs. But in an exhaustive study, Iyú et al³³ showed cangrelor and prasugrel, a thienopyridine related to clopidogrel, act solely on the platelet P2Y₁₂ receptor and not through another GPCR.

Aspirin is a potent antagonist of TBXA₂ production and it blocks platelet aggregation in response to arachidonic acid. However, the response to ADP is not affected by aspirin making aspirin a weak inhibitor of platelet aggregation in vivo. Perhaps, this explains the inability of aspirin to clearly protect hearts from ischemia/reperfusion. Interestingly, Ye and coworkers³⁴ also failed to observe any cardioprotective effect of low- and high-dose aspirin in rats. And in pigs, aspirin appeared to increase the infarct size.³⁵

Cangrelor, which has a very short plasma half-life, required at least 2 hours of continued infusion to protect. We have noted on several occasions that postconditioning drugs such as A_{2B} adenosine receptor agonists must be present for at least 1 hour following the onset of reperfusion for protection to be sustained.^{36,37} We have proposed that the heart must be supported pharmacologically until the ischemically injured myocardium has sufficiently recovered to survive unaided. It is not known why 2 hours rather than 1 hour of treatment was needed for cangrelor to protect but it is unlikely that the drug would be stopped prematurely in patients since platelet inhibitors are routinely continued for many weeks after stent deployment.

It is important to appreciate that cangrelor was protective only if its administration were commenced before reperfusion (Figure 2). There is clinical precedent. Heestermans et al³⁸ noted that the infarct size was reduced and adverse events diminished if the small molecule GPIIb/IIIa antagonist tirofiban was administered before balloon angioplasty rather than after.

There has been some corroborating clinical evidence that antiplatelet treatment is a direct cardioprotectant. Schömig and colleagues³⁹ concluded that supplementation of coronary stenting with abciximab infusion significantly decreased the infarct size compared to fibrinolysis and also diminished adverse clinical events.³⁹ But because the reperfusion technique differed between groups, it was not possible to attribute the benefit to only abciximab. Patti et al¹⁹ compared the effects of 600 and 300 mg loading doses of clopidogrel in patients prior to primary percutaneous coronary intervention for ST-segment elevation myocardial infarction. The larger dose resulted in smaller infarcts, improved cardiac function, and fewer major adverse cardiovascular events. In our rabbits, it took 2 days of high-dose clopidogrel treatment before platelets could be blocked. In humans, the onset of effect is much faster. Zhu et al²² analyzed studies in which platelet reactivity was determined after oral clopidogrel administration. An effect on platelets was seen after 30 minutes and the difference in platelet reactivity between the groups became significant after 60 minutes (18% and 32% inhibition with 300 and 600 mg doses, respectively). Peak inhibition was also significantly higher in the 600 mg group (35% vs 62%). Our hypothesis that cardioprotection is related to the degree of platelet aggregation at the time of reperfusion would explain why the group treated with the higher dose of clopidogrel had

smaller infarcts and better clinical outcomes. Our results with cangrelor indicate that early reperfusion is the critical time for the drug to protect the heart. Indeed, a recent study showed a worse clinical outcome in anticoagulated patients who still had high platelet reactivity at the time of reperfusion despite loading with 600 mg of clopidogrel.⁴⁰ The study suggested that 36% of the patients will not be adequately protected with 600 mg of clopidogrel. If all protection really does occur at reperfusion then it might be better not to give an oral loading dose that may or may not be effective but rather administer a parenteral agent like cangrelor at the appropriate dose just prior to recanalization.

Our observation that adding IPOC to cangrelor treatment provided no additional protection than that seen with cangrelor alone (Figure 2) has important clinical implications. Since most patients with acute myocardial infarction are currently treated with an antiplatelet drug prior to angioplasty and stenting, little benefit would be expected by adding an additional postconditioning intervention. This could explain the rather dismal performance of very promising postconditioning drugs in recent clinical trials.^{20,21} Mochly-Rosen and Grimes²¹ examined the placebo groups in cardioprotection trials over the past 10 years and noted that 3-month mortality has progressively fallen from near 10% in 2000 to less than 4% today. Increased use of antiplatelet drugs over that time period could have played a large role in this improvement.

Staat et al⁴¹ noted that IPOC significantly reduced the infarct size compared to that in patients without postconditioning. The patients were recruited prior to 2005 and approximately half of the patients had not received any antiplatelet agent prior to reperfusion. A retrospective analysis of their clinical database by these investigators clearly reveals the protective property of clopidogrel in man.⁴² Unlike our study, postconditioning along with clopidogrel appeared to give additive protection. But that would be expected if neither one was exerting a maximal effect. Whether their protocol for postconditioning was optimal for human hearts is unknown but half of the clopidogrel-treated patients received only 300 mg which is clearly suboptimal and, as discussed above, even a 600-mg loading dose would only protect about two-thirds of the patients. In contrast, Freixa and colleagues⁴³ were unable to demonstrate a protective effect of IPOC. But all control and postconditioned patients had been pretreated with clopidogrel and a GPIIb/IIIa receptor antagonist, and one might speculate that this regimen had already postconditioned all the patients.

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Figure 1. (A) Platelet aggregation in rabbits after treatment with oral clopidogrel and (B) iv cangrelor. Platelet aggregation was initiated with ADP. (C) Infarct size as a percentage of risk zone after oral treatment with clopidogrel for 2 days or iv cangrelor 10 min before reperfusion. Abbreviations: Wort = wortmannin *p<0.001

Figure 2.

Infarct size as a percentage of risk zone in cangrelor-treated rabbits. The effect on infarction of changing the duration, timing of administration, and dose as well as adding ischemic postconditioning (IPOC) was evaluated. The IPOC group has been previously reported²⁹ and these data were collected by the same investigator who studied the other groups in the figure. It is added for the purpose of comparison. Open symbols depict individual experiments while solid symbols depict group means $\pm$ SEM. * $p < 0.001$

Figure 3.
The effect of signaling antagonists on the anti-infarct action of cangrelor. See text for abbreviations. Format same as in Fig. 2. * $p < 0.001$

Figure 4. Infarct size in isolated hearts. Cangrelor was present in the buffer from 10 min before reperfusion to the end of reperfusion. Format same as in Fig. 2.

Figure 5.

Platelet aggregation in rabbits before (A) and after (B) treatment with aspirin. Platelet aggregation was initiated with arachidonic acid (AA) or ADP. The initial dose of AA was 0.1 mM. If no aggregation was apparent, additional AA was added to make the final concentration 0.35 mM (arrow in A and first arrow in B) and then 0.85 mM (second arrow in B). The triggering effect of the higher dose of arachidonic acid in control platelets was blunted in the presence of aspirin. (C) Infarct size as a percentage of risk zone. Effect of cangrelor is compared to that of aspirin, and heparin. Format same as in Fig. 2. $*p < 0.001$

Table 1

Hemodynamics: Open-chest animals

Groups	Baseline			30 min occlusion			30 min reperfusion			180 min reperfusion		
	H R (bpm)	sys BP (mmHg)	dias BP (mmHg)	HR	sys BP	dias BP	HR	sys BP	dias BP	HR	sys BP	dias BP
Control	270±11	101±3	82±3	268±9	83±3 [*]	67±3 [†]	274±8	88±3 [†]	71±3 [†]	271±8	86±5 [*]	69±4 [*]
Cangrelor (190 min)	279±8	95±3	76±3	273±7	87±2 [†]	71±2	276±9	84±3 [*]	68±2 [†]	284±8	84±2 [*]	65±1 [*]
Cangrelor (130 min)	280±10	97±3	83±3	281±11	87±4	75±4	280±15	93±3	82±4	276±19	85±3	73±2
Cangrelor (70 min)	291±9	101±2	84±2	293±14	87±5	73±5	284±14	84±9	67±9	272±9	82±5 [†]	65±6 [†]
Cangrelor ¼ dose	286±8	96±5	81±5	286±5	80±3	68±3	283±4	81±5	69±5	270±8	81±3	68±4
Cangrelor ⅕ dose	305±11	103±5	92±5	293±7	90±5	81±5	297±6	91±7	79±6	288±6	81±5	70±4 [†]
Cangrelor (10' delay)	289±11	115±4	99±4 [§]	291±6	102±4	87±4	279±3	97±7 [†]	80±6 [†]	281±9	94±2 [†]	76±1 [*]
Cangrelor (190 min) + IPOC	282±6	101±2	80±3	279±4	88±2	73±3	290±3	95±4	79±5	287±11	83±7 [†]	66±7 [†]
Cangrelor + Wort	301±14	107±6	86±5	297±10	91±4	91±4	312±11	103±5	90±5	294±15	96±9	80±9
Cangrelor + LY	301±11	103±3	85±2	285±5	92±4	79±4	289±7	89±4 [†]	76±3	290±11	80±4 [*]	65±3 [*]
Cangrelor + PD	282±10	93±3	76±3	276±9	83±4	69±5	288±9	85±3	71±4	285±5	75±4 [†]	57±6 [†]
Cangrelor + SPT	288±8	102±3	82±1	276±5	92±5	78±7	287±7	90±7 [†]	74±8	279±10	87±6 [†]	70±5
Cangrelor + MRS	275±7	99±2	77±2	278±7	85±3 [†]	69±3	273±8	91±3	78±3	278±7	82±3 [*]	65±2 [†]
Cangrelor + MPG	270±23	102±2	82±2	285±29	89±5 [†]	71±6 [†]	285±26	82±4 [*]	62±4 [*]	262±16	83±5 [*]	64±5 [*]
Cangrelor + 5HD	271±12	102±3	82±2	274±8	82±6 [†]	67±5 [†]	280±10	86±6	69±5	278±6	79±2 [†]	63±1 [†]
Clopidogrel	283±4	104±5	86±5	290±7	95±3	78±3	294±7	97±3	79±2	297±15	91±3 [†]	73±4
Clopidogrel + Wort	310±8	97±3	79±4	283±5	102±2	88±2	283±4	98±3	84±2	267±6	92±2	77±2
Clopidogrel + MRS	296±4	99±3	83±2	277±4	91±2	77±2	270±6	88±4 [†]	76±4	280±6	84±2 [*]	68±3 [*]
Aspirin	326±9	104±4	89±4	297±5	92±5 [*]	79±4 [†]	281±5	89±4 [*]	76±3 [†]	291±6	91±4 [*]	78±5 [†]
Heparin	297±6	99±3	80±4	293±7	90±5 [†]	71±4 [†]	285±6	87±3 [†]	69±3 [†]	278±7	86±4 [*]	65±4 [*]

Abbreviations: BP = blood pressure dias = diastolic 5HD = 5-hydroxydecanoate HR = heart rate IPOC = ischemic postconditioning LY = LY294002 MPG = 2-mercaptopropionylglycine MRS = MRS1754 PD = PD98059 SPT = 8-sulphonyltheophylline sys = systolic wort = wortmannin

Statistical significance of difference between experimental and control groups at baseline:

[§] p<0.01

Statistical significance of difference between baseline and other time point in any given group:

[†] p<0.05
[‡] p<0.01
^{*} p<0.001

Table 2

Platelet aggregation

	Aggregation curve area after 5 min, % of control		
	ADP, 5 μ M	ADP, 10 μ M	Arachidonic acid, 0.35 mM
Control	100	100	100
Cangrelor, full dose		6 $\pm$ 3 *	
Cangrelor, quarter dose		28 $\pm$ 8 *	
Cangrelor, eighth dose		50 $\pm$ 5 *	
Clopidogrel		22 $\pm$ 4 *	
Aspirin	90 $\pm$ 6		18 $\pm$ 10 *

* p<0.001 vs. Control

Table 3

Infarct data

Group	N	BW (kg)	HW (g)	Risk Zone (g)	Infarct Size (g)	IS/RZ (%)
Control	19	2.3±0.0	7.6±0.2	1.30±0.06	0.050±0.04	37.9±1.8
Cangrelor (190 min)	10	2.5±0.0	7.8±0.2	1.41±0.10	0.28±0.04 [‡]	19.4±2.1 [‡]
Cangrelor (130 min)	6	2.4±0.1	8.1±0.3	1.48±0.09	0.34±0.05	22.3±2.2 [‡]
Cangrelor (70 min)	6	2.6±0.1	7.7±0.2	1.48±0.10	0.45±0.05	30.5±3.2
Cangrelor (10' delay)	5	2.8±0.1 [‡]	8.5±0.2	1.56±0.15	0.72±0.08	45.9±1.4
Cangrelor ¼ dose	6	2.4±0.0	7.6±0.4	1.40±0.15	0.34±0.04	24.0±1.6 [‡]
Cangrelor ⅓ dose	6	2.4±0.0	7.1±0.3	1.28±0.12	0.51±0.10	39.5±5.0
IPOC [§]	10	2.4±0.1	7.4±0.2	1.23±0.10	0.25±0.03 [*]	19.8±1.8 [‡]
Cangrelor (190 min) + IPOC	5	2.2±0.0	6.9±0.2	1.35±0.04	0.33±0.03	24.1±1.3 [‡]
Cangrelor + Wort	5	2.4±0.1	8.5±0.2	1.64±0.15	0.67±0.11	39.7±3.1
Cangrelor + LY	5	2.2±0.0	7.7±0.2	1.38±0.33	0.69±0.09	42.0±2.6
Cangrelor + PD	5	2.4±0.0	8.5±0.3	1.84±0.14 [‡]	0.70±0.06	38.1±2.4
Cangrelor + SPT	5	2.4±0.0	8.4±0.1	1.67±0.07	0.63±0.03	38.1±1.8
Cangrelor + MRS	6	2.2±0.0	6.4±0.2 [*]	1.16±0.06	0.47±0.06	40.3±3.5
Cangrelor + MPG	5	2.5±0.0	8.6±0.2	1.55±0.15	0.66±0.09	42.2±2.3
Cangrelor + SHD	5	2.6±0.1 [‡]	8.6±0.2	1.50±0.19	0.61±0.10	39.9±2.4
Clopidogrel	6	2.5±0.1	8.2±0.2	1.24±0.11	0.23±0.04 [‡]	18.0±2.4 [‡]
Clopidogrel + Wort	6	2.4±0.1	7.6±0.3	1.29±0.11	0.55±0.08	41.3±3.0
Clopidogrel + MRS	6	2.3±0.0	7.2±0.3	1.28±0.12	0.50±0.06	38.9±1.4
Aspirin	7	2.4±0.1	6.9±0.3	1.12±0.05	0.34±0.08	29.0±5.9
Heparin	6	2.6±0.1 [‡]	9.1±0.2 [‡]	1.33±0.04	0.62±0.07	46.4±4.2

Abbreviations: see Table 1 BW = body weight HW = heart weight IS/RZ = ratio of infarct size to risk zone volume N = number of animals

Statistical significance of difference between experimental group and respective control:

[‡] p<0.05

*
p<0.01

p<0.001

^gThis group is included for the purpose of comparison. It has already been reported.²⁹