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SHORT COMMUNICATION

# Cangrelor-Mediated Cardioprotection Requires Platelets and Sphingosine Phosphorylation

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**Abstract** In animal models platelet $P2Y_{12}$ receptor antagonists put the heart into a protected state, not as a result of suppressed thrombosis but rather through protective signaling, similar to that for ischemic postconditioning. While both ischemic postconditioning and the $P2Y_{12}$ blocker cangrelor protect blood-perfused hearts, only the former protects buffer-perfused hearts indicating that the blocker requires a blood-borne constituent or factor to protect. We used an anti-platelet antibody to make thrombocytopenic rats to test if that factor resides within the platelet. Infarct size was measured in open-chest rats subjected to 30-min ischemia/2-h reperfusion. Infarct size was not different in thrombocytopenic rats showing that preventing aggregation alone is not protective. While ischemic preconditioning could reduce infarct size in thrombocytopenic rats, the $P2Y_{12}$ inhibitor cangrelor could not, indicating that it protects by interacting with some factor in the platelet. Ischemic preconditioning is known to require phosphorylation of sphingosine. In rats treated with dimethylsphingosine to block sphingosine kinase, cangrelor was no longer protective. Thus cangrelor's protective mechanism appears to also involve sphingosine kinase revealing yet another similarity to conditioning's mechanism.

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Loading patients presenting with acute myocardial infarction with a platelet inhibitor prior to recanalization has become standard of care. It was assumed that the improved outcomes were the result of preventing intravascular thrombi, but we recently found that these platelet antagonists triggered an anti-infarct effect in animal hearts that was actually the result of protective cellular signal transduction pathways independent of any anti-thrombotic effect [1]. We also noted that neither ischemic preconditioning (IPC) [2] nor ischemic postconditioning (IPOC) [1] offered any additional protection to animals treated with the platelet $P2Y_{12}$ blocker cangrelor, again suggesting similar mechanisms of action.

We have studied the effect of the $P2Y_{12}$ inhibitors clopidogrel, ticagrelor, and cangrelor in rats [2], rabbits [1] and mice [3], and noted potent infarct-sparing properties when they were given before ischemia or even when started just prior to reperfusion. While protection from all 3 of the above agents was seemingly the result of intracellular signaling, it was a mystery how these agents triggered those signaling pathways. When we studied cangrelor in a blood-free, isolated rabbit [1] or mouse [3] heart, it was no longer able to limit infarction. That would suggest a formed element in the blood was involved in the trigger mechanism. The present study therefore examines whether platelets were that critical factor by testing if cangrelor can protect a thrombocytopenic rat. To further explore the mechanism of cangrelor's protection we examined whether phosphorylation of sphingosine might be involved in the protection since platelets are known to release sphingosine upon activation and phosphorylation of sphingosine has been shown to be an essential event in the conditioning pathway [4–7].

## Methods

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 [8].

### Surgical Preparation

Male Sprague-Dawley rats weighing 400–600 g were anesthetized with intraperitoneal pentobarbital, 100 mg/kg. The trachea was intubated and the lungs were inflated with 100 % oxygen. A carotid artery was cannulated for measurement of blood pressure and withdrawal of blood. A jugular vein was also cannulated for administration of drugs. After a left thoracotomy exposed the heart, a snare was placed around the left anterior descending coronary artery. All hearts were exposed to 30-min coronary artery occlusion and 2-h reperfusion.

### Infarct Area and Risk Zone

As previously described [1] the heart was excised at the end of the experiment and mounted on a Langendorff apparatus. The coronary artery was re-occluded, and 2–9 μm fluorescent microspheres (Microgenics, Freemont, CA) were injected into the aortic perfusate to demarcate ischemic zone. The heart was then frozen and cut into two-mm thick slices from apex to base which were then incubated in 1 % triphenyltetrazolium chloride (GFS Chemicals, Powell, OH) in pH 7.4 buffer at 37 °C to distinguish living (stained) from infarcted (unstained) tissues. Risk zone and infarcted regions were measured by planimetry by a person blinded to the treatment. Volumes were calculated and infarct size is expressed as a percentage of risk zone.

Platelet aggregation was determined by measuring impedance with a whole blood aggregometer (Chrono-log, Havertown, PA). Aggregation was initiated by addition of ADP to produce a final concentration of 10 μM. Area under each aggregation curve was measured and averaged for each group.

### Anti-Thrombocyte Serum Studies

Rats were treated with an intraperitoneal injection of 0.5 mL rabbit anti-rat anti-thrombocyte serum (ATS) (AIA51440, Accurate Chemical and Scientific Corp., Westbury, NY) or a comparable volume of saline and studied 24 h later. Venous blood was obtained from the rat's tail vein for measurement of platelet count (Automated Hematology Analyzer, ABX Micros 60-CT, ABX, Montpellier, France). The first group was a control group subjected to only ischemia/reperfusion. In the second, rats were treated with only ATS. The third was treated with cangrelor (60 μg/kg bolus in saline 10 min before reperfusion followed by 6 μg/kg/min for the duration of the study). The fourth group was treated with both ATS and cangrelor. The fifth group underwent IPC with 3 cycles of 5-min coronary occlusion/5-min reperfusion before the 30-min occlusion. The last group was treated with ATS and IPC.

### Sphingosine Studies

N,N-Dimethylsphingosine (DMS) (Sigma-Aldrich, St. Louis, MO) which blocks the phosphorylation of sphingosine by sphingosine kinase was administered as an intravenous bolus, 0.33 mg/kg, 15 min after coronary occlusion. One group of rats undergoing ischemia/reperfusion was treated with only DMS, while another group was additionally treated with cangrelor as detailed above.

### Statistics

Infarct sizes were analyzed by one-way ANOVA. Post hoc testing was done with Student-Newman-Keuls test. The control and cangrelor groups are larger because control and cangrelor experiments were intermixed with other interventions performed serially to be certain conditions had not changed over the course of the study. As no trends were seen all groups were compared to that single large group. Changes in platelet counts before and after ATS were analyzed with a paired t-test. A $p$ value of $<0.05$ was considered to be significant.

## Results

A total of 55 rats were used for these experiments. There were no differences in either heart rate or mean blood pressure between the 8 experimental groups before coronary occlusion (data not shown). In all groups blood pressure fell during the coronary occlusion followed by partial recovery after onset of reperfusion.

### Platelet Depletion

Platelet counts prior to intraperitoneal administration of either saline or ATS averaged $574 \pm 24 \times 10^3/\mu L$. The average platelet count 1 day after treatment with 0.5 mL ATS was $26 \pm 4 \times 10^3/\mu L$. This represented an average decrease of 95.4 ± 0.7 % which was statistically very significant ($p < 0.001$). Changes in white blood cell count and hematocrit were minimal. As in prior reports [1] cangrelor blocked platelet aggregation in rats with normal platelet counts in response to ADP by ~90 %. Aggregation was not detected in rats treated with ATS.

Infarct size data are presented in Fig. 1. As previously documented [1–3] cangrelor was very protective and significantly decreased infarction of the risk zone ($p < 0.001$).

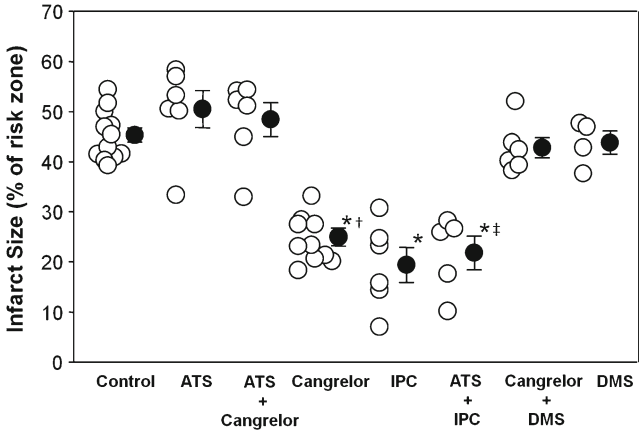


**Fig. 1** Infarct size as a percentage of risk zone for control rats and those treated with either anti-thombocyte serum (ATS), cangrelor, or N,N-dimethylsphingosine (DMS). Empty circles represent individual infarct sizes, and filled circles represent group means ± SEM. Infarct sizes were not different among the control, DMS, and cangrelor + DMS groups. Statistical significance of group differences: between Control and either Cangrelor, ischemic preconditioning (IPC), or ATS + IPC, $*p < 0.001$; between Cangrelor and either ATS + Cangrelor or Cangrelor + DMS, $\dagger p < 0.001$; between ATS and ATS + IPC, $\ddagger p < 0.001$

However, when cangrelor was administered to rats pretreated with ATS, the anti-platelet agent's protective effect was absent. ATS by itself had no effect on infarct size. During surgery on rats pre-treated with ATS we noted a coagulopathy similar to that seen following cangrelor exposure. The fact that platelet depletion alone did not reduce infarct size argues against the hypothesis that cangrelor reduces infarct size by preventing intravascular thrombi.

IPC was as protective as cangrelor. Notably the cardioprotective effect of IPC was not affected by platelet depletion. Therefore, the platelet seems to be the missing component in the blood that prevents cangrelor from protecting buffer-perfused hearts.

### Sphingosine

Infarct data for the 2 unique groups in this series of experiments are again presented in Fig. 1. DMS blocked cangrelor's protection. Note that DMS alone had no effect on infarct size when compared to that in untreated control rats.

## Discussion

$P2Y_{12}$ receptor antagonists are used in primary angioplasty to attenuate platelet aggregation and thus inhibit thrombus formation. But there is a second action of $P2Y_{12}$ antagonists which may be equally or even more important and is greatly under-appreciated. The three antagonists that we have studied to date, clopidogrel, ticagrelor, and cangrelor, all make the myocardium very resistant to infarction [1–3]. When oral agents clopidogrel and ticagrelor are administered to ischemic hearts before coronary occlusion, infarct size is nearly halved [1, 2]. Administration of cangrelor shortly before the onset of reperfusion results in cardioprotection. Surprisingly, the agent's anti-aggregatory property can be separated from its cardioprotective property. Blockade of signaling pathways used by IPOC prevented cangrelor's anti-infarct effect but not its anti-aggregatory effect [1, 2]. Interestingly, giving cangrelor several minutes after reperfusion renders it ineffective as an anti-infarct agent [1], as has been observed with other postconditioning-mimetics [9], and no doubt explains why loading the patient with the drug prior to recanalization is so important.

The target for $P2Y_{12}$ inhibitors to prevent platelet aggregation is clearly the $P2Y_{12}$ receptors on the platelet. Since 3 structurally different $P2Y_{12}$ antagonists all are powerful cardioprotectants, it seems likely that a $P2Y_{12}$ receptor is the target for cardioprotection as well. But is that the receptor on the platelet? We addressed this question by examining the effect of cangrelor on infarction in a buffer-perfused rabbit heart [1]. These hearts could still be protected by either IPC [10] or IPOC [11]. Yet, unlike the case in in situ blood-perfused hearts, cangrelor could not protect them. Therefore some constituent of blood was needed for cardioprotection. This observation was recently confirmed in isolated mouse hearts in which a wide range of concentrations of cangrelor were all ineffective in reducing infarct size [3].

The present report addresses the platelet question directly. We used ATS which selectively depletes platelets. In our experiments ATS decreased platelet counts by 96 %. Cangrelor could not protect the hearts of platelet-depleted rats. Yet the cardioprotective signaling pathways were still intact as IPC continued to protect. This is strong evidence that the blood element required for cangrelor's cardioprotection is indeed the platelet.

Activated platelets are known to release the cardioprotective chemical sphingosine-1-phosphate (S1P). The platelet cannot synthesize sphingosine de novo, but platelets can incorporate extracellular sphingosine which is then converted to S1P by sphingosine kinase in the platelets and stored [12]. S1P has been reported to be an important trigger for the protection seen in IPC [4]. Binding of S1P to the $S1P_1$ receptor initiates downstream activation of ERK 1/2, while binding the $S1P_3$ receptor leads to activation of phosphatidylinositol 3-kinase and Akt. Thus S1P triggers the reperfusion injury survival kinase or RISK pathway [6, 7] that is well known to produce the protected state associated with IPC and IPOC [13]. S1P also triggers the protective survivor activating factor enhancement or SAFE pathway. Ligand binding of the $S1P_2$ receptor activates ERK 1/2 and subsequently STAT3 [6, 7]. Sphingosine intermediates are also involved in cardioprotection mediated by TNF-$\alpha_1$ [5]. This redundancy provides potential for robust protection as is seen in IPC.

Because we have found that protection from $P2Y_{12}$ inhibitors seems to depend on the same signaling components as

IPC and IPOC, it seemed likely that sphingosine would be involved in cangrelor's protective mechanism as well. We prevented the phosphorylation of sphingosine with DMS, a blocker of sphingosine kinase. DMS will block the protective effect of IPC [4] and in the present experiment it also blocked cangrelor's ability to protect the reperfused rat heart (see figure). In the case of IPC S1P must come from the heart rather than the blood since DMS blocked IPC's protection in Krebs buffer-perfused hearts [4]. Whether it was the heart or the platelet that was producing the S1P that was required to protect the cangrelor-treated hearts cannot be so easily determined since cangrelor is ineffective in buffer-perfused hearts. Thus cangrelor could either cause platelets to produce S1P which then triggers the conditioned state or alternatively cangrelor-treated platelets may trigger the conditioned state independent of sphingosine which then causes ischemic myocardium to release S1P. What this experiment does show, however, is yet another similarity between the signaling pathways involved in the protection from cangrelor and that from IPOC.

**Compliance with Ethical Standards**

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## References

1. Yang X-M, Liu Y, Cui L, Yang X, Liu Y, Tandon N, et al. Platelet $P2Y_{12}$ blockers confer direct postconditioning-like protection in reperfused rabbit hearts. J Cardiovasc Pharmacol Ther. 2013;18:251–62.
2. Yang X-M, Cui L, Alhammouri A, Downey JM, Cohen MV. Triple therapy greatly increases myocardial salvage during ischemia/reperfusion in the in situ rat heart. Cardiovasc Drugs Ther. 2013;27:403–12.
3. Bell RM, Sivaraman V, Kunuthur SP, Cohen MV, Downey JM, Yellon DM. Cardioprotective properties of the platelet $P2Y_{12}$ receptor inhibitor, cangrelor: protective in diabetics and reliant upon the presence of blood. Cardiovasc Drugs Ther 2015;29:415–8.
4. Jin Z-Q, Goetzl EJ, Karliner JS. Sphingosine kinase activation mediates ischemic preconditioning in murine heart. Circulation. 2004;110:1980–9.
5. Lecour S, Smith RM, Woodward B, Opie LH, Rochette L, Sack MN. Identification of a novel role for sphingolipid signaling in TNFα and ischemic preconditioning mediated cardioprotection. J Mol Cell Cardiol. 2002;34:509–18.
6. Knapp M Cardioprotective role of sphingosine-1-phosphate. J Physiol Pharmacol. 2011;62:601–7.
7. Somers SJ, Frias M, Lacerda L, Opie LH, Lecour S. Interplay between SAFE and RISK pathways in sphingosine-1-phosphate-induced cardioprotection. Cardiovasc Drugs Ther. 2012;26:227–37.
8. National Research Council. Guide for the Care and Use of Laboratory Animals. 7th ed. Washington, D.C.:National Academy Press;1996.
9. Hausenloy DJ, Duchen MR, Yellon DM. Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. Cardiovasc Res. 2003;60:617–25.
10. Sandhu R, Diaz RJ, Wilson GJ. Comparison of ischaemic preconditioning in blood perfused and buffer perfused isolated heart models. Cardiovasc Res. 1993;27:602–7.
11. Yang X-M, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005;100:57–63.
12. Tani M, Sano T, Ito M, Igarashi Y. Mechanisms of sphingosine and sphingosine 1-phosphate generation in human platelets. J Lipid Res. 2005;46:2458–67.
13. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res. 2004;61:448–60.