

Platelets Protect Cardiomyocytes from Ischemic Damage

Tony G. Walsh¹ Alastair W. Poole¹

¹School of Physiology, Pharmacology, and Neuroscience, University of Bristol, Bristol, United Kingdom

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Address for correspondence Alastair W. Poole, PhD, School of Physiology, Pharmacology, and Neuroscience, Biomedical Sciences Building, University of Bristol, Bristol BS8 1TD, United Kingdom (e-mail: a.poole@bristol.ac.uk).

Abstract

Platelets are classically known for their roles in bleeding control and occlusive thrombus formation causing ischemic tissue damage. Recently, nonclassical roles for platelets have been described, many of which may be mediated by the heterogeneous cargo that platelets secrete from granular stores upon activation. Using an *in vitro* model of ischemic injury to ventricular cardiomyocytes, we observed that platelets, through secreted factors, delayed the rate of cardiomyocyte death during ischemia. This protective effect appeared independent of platelet dense granule cargo, but required α -granule components stromal cell-derived factor-1 α and transforming growth factor- β 1. Protein kinase C activity within cardiomyocytes was responsible for mediating the protective signals initiated by the released platelet cargo. Importantly, pretreating platelets with a P2Y₁₂ antagonist, but not the cyclooxygenase inhibitor aspirin, substantially attenuated this protective effect. These findings therefore reveal a paradoxically protective role for platelet activation during cardiac ischemia and could have important implications for the use of antiplatelet therapeutics in the management of myocardial infarction.

Keywords

- ▶ platelets
- ▶ secretion
- ▶ thrombosis
- ▶ cardiomyocytes
- ▶ ischemia

Introduction

Platelets are the guardians of hemostasis, but are also established mediators of pathological thrombosis causing myocardial infarction and stroke. More recently, novel unconventional roles for platelets have emerged with the repertoire extending to numerous physiological and pathological roles within the body. From a physiological context, platelets are crucial for tissue regeneration,¹ wound healing,² and protecting from septic shock,³ but pathologically, they have established roles in atherogenesis,⁴ tumor stability,⁵ and metastasis.⁶ Key to this functional heterogeneity is the armory of biological cargo stored within distinct secretory platelet granules, namely, α -, dense, and lysosomal granules, the contents of which are released upon platelet activation. Platelet α -granules represent the most abundant and heterogeneous granules in terms of cargo, containing over 300 proteins released upon activation.⁷ These include growth factors, chemokines, cytokines, clotting factors, and adhesive proteins,

and are widely recognized for eliciting this broad functionality of platelets.^{8,9}

In this study, we assessed the direct effects of released cargo from activated platelets on isolated ventricular cardiomyocytes during ischemic injury. Undoubtedly, platelets, through occlusive thrombosis of coronary arteries, trigger ischemic injury and furthermore they have causative roles in the pathogenesis of myocardial reperfusion injury, particularly through their ability to recruit and activate inflammatory cells.^{10,11} Interestingly, cardioprotective roles for platelets have also been described, effects which appear to be indirectly mediated by the endothelium.^{12,13} However, there is no evidence assessing direct, paracrine effects of platelet-derived factors on cardiomyocytes during ischemic injury. Recently, heterocellular signaling pathways between endothelial-derived growth factors (neuregulin-1) and ErbB4 receptors on cardiomyocytes have been identified and we therefore hypothesized that vascular endothelial denudation, which initiates platelet activation, represents a likely conduit for soluble platelet-derived factors to interact

with the myocardium directly during coronary thrombosis.¹⁴ Our study identifies a novel role for platelets in preconditioning cardiomyocytes to ischemic damage. We show that secreted platelet factors alter the tolerance of cardiomyocytes to ischemic injury by delaying the rate of cardiomyocyte death and we further explore the mechanism behind this protective effect. Importantly, we observed that blocking platelet P2Y₁₂, a clinical target of platelet-dependent thrombosis, obscures this nonconventional aspect of platelet function. This is in contrast to aspirin, also used clinically in the management of myocardial infarction, which preserves the cardioprotective effects of platelets and could therefore have future implications for antiplatelet strategies in the management of heart attacks.

Methods

Mice

All animal studies were approved by the local research ethics committee at the University of Bristol. Mice were bred and maintained in the University of Bristol animal facility in accordance with the UK Home Office regulations and Animals (Scientific Procedures) Act of 1986 (PPL No: 3003445). Male, C57BL/6 wild-type (WT) mice were from Harlan Laboratories (Bicester, UK). Unc13d^{flm} mice on a C57BL/6 background were from the Mutant Mouse Regional Resource Center (University of California, United States).

Platelet Preparation

Eight- to 20-week old mice were sacrificed by a gradual rise in CO₂ and blood was drawn from the inferior vena cava into a syringe containing 4% trisodium citrate (1:10 v/v). Washed platelets were prepared as previously described,¹⁵ resuspended in modified HEPES-Tyrode's buffer (10 mM HEPES, 145 mM NaCl, 1 mM MgCl₂, 3 mM KCl, 5 mM Glucose, pH 7.3) without apyrase addition and allowed to recover for 30 minutes at 30°C. Immediately prior to stimulation, 1 mM CaCl₂ was added to platelets.

Platelet Activation: Releasate Isolation and α -Granule Secretion

For platelet experiments involving pharmacological inhibitors, platelets at 2×10^8 /mL were pretreated for 10 minutes prior to stimulation with vehicle (HEPES-Tyrode's) or 10 μ g/mL collagen-related peptide (CRP; from Prof. Richard Farndale, University of Cambridge, UK) for 15 minutes at 37°C. Inhibitors AR-C 66096 (Tocris Bioscience, Bristol, UK) and aspirin (Sigma-Aldrich, Poole, UK) were used to block P2Y₁₂ and cyclooxygenase, respectively. For releasate isolation, 140 nmol/L PGE₁ (Sigma-Aldrich) was added poststimulation and platelets were pelleted by centrifugation at 580g for 10 minutes. The supernatant containing released platelet factors (henceforth referred to as "platelet releasate") was removed and subjected to 2 \times pulse centrifugations to clarify any remaining debris and the soluble releasate was used to pretreat cardiomyocytes. Fractionation of the platelet releasates using 3-kDa molecular weight cutoff filters was performed as per manufacturer's instructions (Millipore, Hertfordshire, UK). In brief, 500 μ L of

platelet releasate was centrifuged at 14,000g for 20 minutes. This yielded approximately 400 μ L of filtered releasate, with approximately 100 μ L of retained releasate, which was adjusted to 400 μ L with HEPES-Tyrode's. To measure α -granule secretion, platelets, poststimulation, were incubated with 2.5 μ L FITC-labeled antimouse CD62P (Emfret Analytics, Würzburg, Germany) for 5 minutes, then fixed in 1% paraformaldehyde for 30 minutes and analyzed using a FACS Canto II flow cytometer (Becton Dickinson, New Jersey, United States). Samples were analyzed with FACSDiva software where a total of 10,000 gated events per sample were collected.

Ventricular Cardiomyocyte Isolation

Male, adult (9–14 weeks) WT mice were intraperitoneally injected with 400 U of heparin prior to schedule 1 by cervical dislocation. Hearts were rapidly excised and placed in a nominally (0.05 mM) CaCl₂-free HEPES-Tyrode's perfusion buffer (henceforth referred to as perfusion buffer—130 mM NaCl, 5.4 mM KCl, 0.4 mM NaH₂PO₄, 4.2 mM HEPES, 10 mM Glucose, 1.4 mM MgCl₂, 20 mM Taurine, 1 mM Creatine, 10 mM 2,3-butanedione monoxime, and pH 7.4). The aorta was cannulated and the heart perfused retrograde, in a noncirculating mode on a Langendorff apparatus in CaCl₂-free perfusion buffer at a flow rate of 3 mL/min for 4 minutes at 37°C. The buffer was switched to a myocyte digestion buffer containing 0.7 mg/mL collagenase type II (Worthington Biochemicals, New Jersey, United States) and 0.1 mg/mL Protease XIV (Sigma-Aldrich) in 0.05 mM CaCl₂-containing perfusion buffer for 15 minutes at 37°C until the heart appeared pale and swollen. Atria were removed and digested ventricles were minced and triturated in perfusion buffer containing 5% fetal calf serum to inactivate proteases, and then sequentially filtered through a 200- μ m nylon gauze (Millipore) resulting in four isolates. Cardiomyocytes were isolated by centrifugation for 1 minute at 60g, resuspended in 0.1 mM CaCl₂ containing perfusion buffer, and visualized at 100 \times on a light microscope (Swift M4000-D, California, United States). Isolate 1 contained mostly round, hypercontracted and nonviable cardiomyocytes which were discarded. Isolates 2 to 4 were pooled and Ca²⁺ was gradually restored in a step-wise fashion to 1 mM over 30 minutes. The isolation typically yielded 60% Ca²⁺ tolerant cardiomyocytes.

In vitro Myocardial Ischemia Viability Assay

The assay setup was based on the established "ischemic pelleting" model, with minor modifications.¹⁶ For experiments involving pharmacological inhibitors, cardiomyocytes were pretreated for 30 minutes with indicated inhibitors: AMD3100 (CXCR4); SB431542 (TGFB1), and BIM I (protein kinase C [PKC])—all from Tocris. Cardiomyocytes were then treated with platelet releasates, diluted 1:2 with 1 mM Ca²⁺ containing perfusion buffer or recombinant proteins for 10 minutes at 37°C. Cardiomyocytes were then allowed to recover for 10 minutes in perfusion buffer prior to ischemic pelleting. With the cardiomyocytes pelleted, precisely 450 μ L buffer was removed from each sample, which was carefully layered with 200 μ L mineral oil to exclude gaseous

exchange and initiate ischemia, that is, $T = 0$ minutes. Every 30 minutes (for up to 240 minutes), a 2.5 μL sample was removed from the pellet and resuspended in 50 μL reperfusion buffer (perfusion buffer containing 3 mM Amytal – Sigma-Aldrich) for 4 minutes. The sample was mixed with an equal volume of counting media (perfusion buffer containing 1% glutaraldehyde and 0.4% trypan blue) allowing detection of viable (trypan blue excluded) and nonviable (trypan blue stained) cardiomyocytes. Images were acquired with a mounted digital microscope camera (AmScope, California, United States) at 100 \times magnification, with eight random fields of view per sample at every time point (equating to ~ 250 –300 cardiomyocytes/time point). This method allowed for a maximum of three treated samples per isolation.

Western Blotting

Cardiomyocytes were lysed in buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100) containing protease and phosphatase inhibitor cocktail sets (Roche, UK) and harvested as previously described.¹⁷ Equal amounts of protein (15 μg /sample) were resolved by electrophoresis on 10% Tris-glycine gels, transferred onto Immobilon PVDF membranes (Millipore), blocked and probed with specific antibodies: Phospho-(Ser) PKC substrate, phospho-Erk1/2, (Thr202/Tyr204), Erk1/2, phospho-Akt, (Ser 473), and Akt antibodies (all 1:1,000 dilution) were from Cell Signaling Technology (Massachusetts, United States) and mouse-anti-GAPDH (1:2,000 dilution) was from Santa Cruz Biotechnology (Heidelberg, Germany). HRP-conjugated secondary antibodies (antimouse and antirabbit, both 1:10,000 dilution) were from GE Healthcare (Buckinghamshire, UK).

Statistical Analysis

All data were analyzed using GraphPad Prism 7 software (GraphPad Software Inc., California, United States). Data are presented as mean \pm standard deviation with the indicated number of independent experiments. Statistical analysis was determined using one- or two-way ANOVA with Bonferroni's post hoc test. $p < 0.05$ was considered statistically significant.

Results

Released Factors from Platelet Granules Protect Cardiomyocytes during Ischemia

During myocardial infarction, platelets release bioactive molecules exerting autocrine/paracrine effects causing occlusive thrombosis of coronary arteries, with subsequent ischemic damage to cardiac tissue.¹⁸ Our initial observations demonstrated that biomolecules released from platelets activated with 10 $\mu\text{g}/\text{mL}$ CRP, a ligand for the platelet-specific collagen receptor, glycoprotein (GP) VI, could delay the rate of cardiomyocyte death during ischemic injury compared with controls (\rightarrow Fig. 1A, B). The effect was not immediate, but was consistently observed between 60 and 180 minutes post ischemia onset and lost between 210 and 240 minutes. Dose response analysis identified 10 $\mu\text{g}/\text{mL}$ CRP to induce maximal platelet secretion, by means of surface exposure of the α -granule protein, CD62P (\rightarrow Fig. S1A, supplementary figure available in the online version only). Importantly, control experiments with CRP alone did not alter the cardiomyocyte response to ischemic cell death (\rightarrow Fig. S1B, supplementary figure available in the online version only). Platelet granules represent the most abundant sources for released platelet cargo and *Unc13d*^{fl^{lox}} mice, which lack the

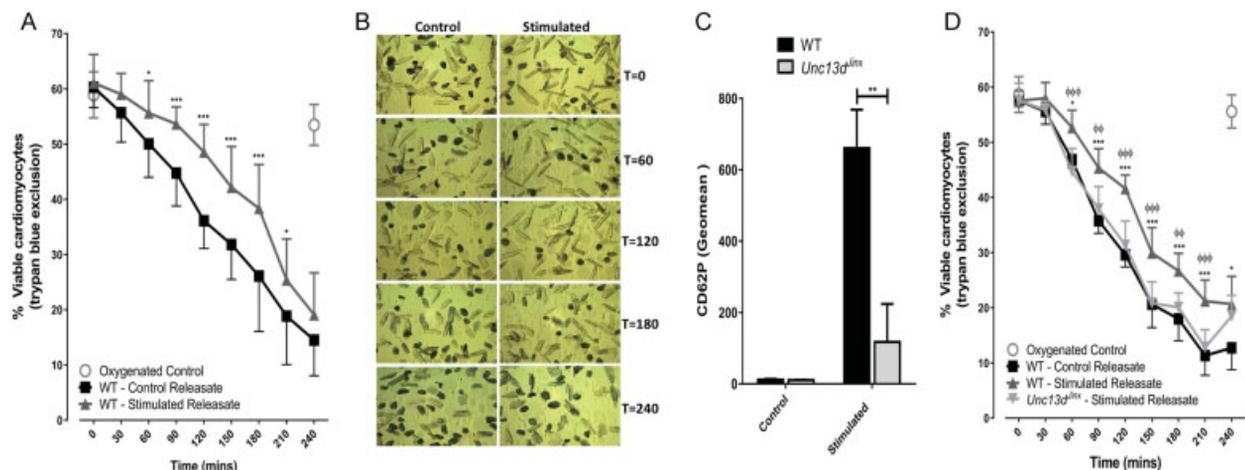


Fig. 1 Factors released from platelet secretory vesicles protect cardiomyocytes from ischemic injury. (A and D) Cardiomyocytes from wild-type (WT) hearts preconditioned with released factors from vehicle-treated (control) and collagen-related peptide (CRP)-treated (stimulated) platelets from WT or *Unc13d*^{fl^{lox}} mice (as indicated) were subjected to ischemic injury using a cell pelleting method. Samples from the pellet were analyzed for cell viability every 30 minutes, for up to 240 minutes, with nonischemic (oxygenated control) samples monitored at 0- and 240-minute time points. Data are mean \pm SD ($n = 7$ for (A), $n = 5$ for (D)), (A) $*p < 0.05$, $***p < 0.001$ versus WT-controlled releasate and (D) $\Phi\Phi p < 0.01$, $\Phi\Phi\Phi p < 0.001$ versus *Unc13d*^{fl^{lox}} stimulated releasate. (B) Representative images of trypan blue stained and nonstained cardiomyocytes at 60-minute intervals throughout ischemia assay (0–240 minutes). (C) Washed platelets ($2 \times 10^8/\text{mL}$) from WT and *Unc13d*^{fl^{lox}} mice were treated with vehicle control (Tyrode's) or 10 $\mu\text{g}/\text{mL}$ CRP and monitored for α -granule secretion by CD62P surface expression. Data are mean \pm SD; $n = 3$, $**p < 0.01$. Two-way ANOVA with Bonferroni post hoc test for (A), (C), and (D).

vesicle priming protein Munc13-4, have proved useful in dissecting functional roles for platelet granules.¹⁹ We confirmed platelet α -granule secretion with CRP was reduced by approximately 82% in Munc13-4^{-/-} platelets (**Fig. 1C**). Subsequent experiments demonstrated that the cardioprotective effects of stimulated platelet releasates from these mice were completely lost in comparison to wild-type (WT) stimulated platelet releasates, suggesting the active component(s) in the releasate are of granular origin (**Fig. 1D**).

Platelet Dense Granules Are Not the Source of Cardioprotective Cargo

To discriminate between dense and α -granule cargo, we took advantage of the fact that cargo in dense granules has generally very low molecular mass, whereas that in

α -granules is principally peptide based and is of much higher average molecular mass. We therefore fractionated the stimulated releasates from WT platelets into less than and more than 3 kDa fractions. Notably, the less than 3 kDa fraction did not delay the kinetics of cardiomyocyte death during ischemia, whereas the more than 3 kDa fraction did significantly delay cardiomyocyte death in a manner similar to unfiltered releasates from stimulated WT platelets (**Fig. 2A**). To ensure the fractionation procedure effectively separated dense and α -granule components, we demonstrated that a significant portion (~78%) of released adenosine triphosphate (ATP) is present in the less than 3 kDa fraction (**Fig. S2A**, supplementary figure available in the online version only). These data support the hypothesis that α -granule content, but not dense granule content, mediates the cardioprotective effect.

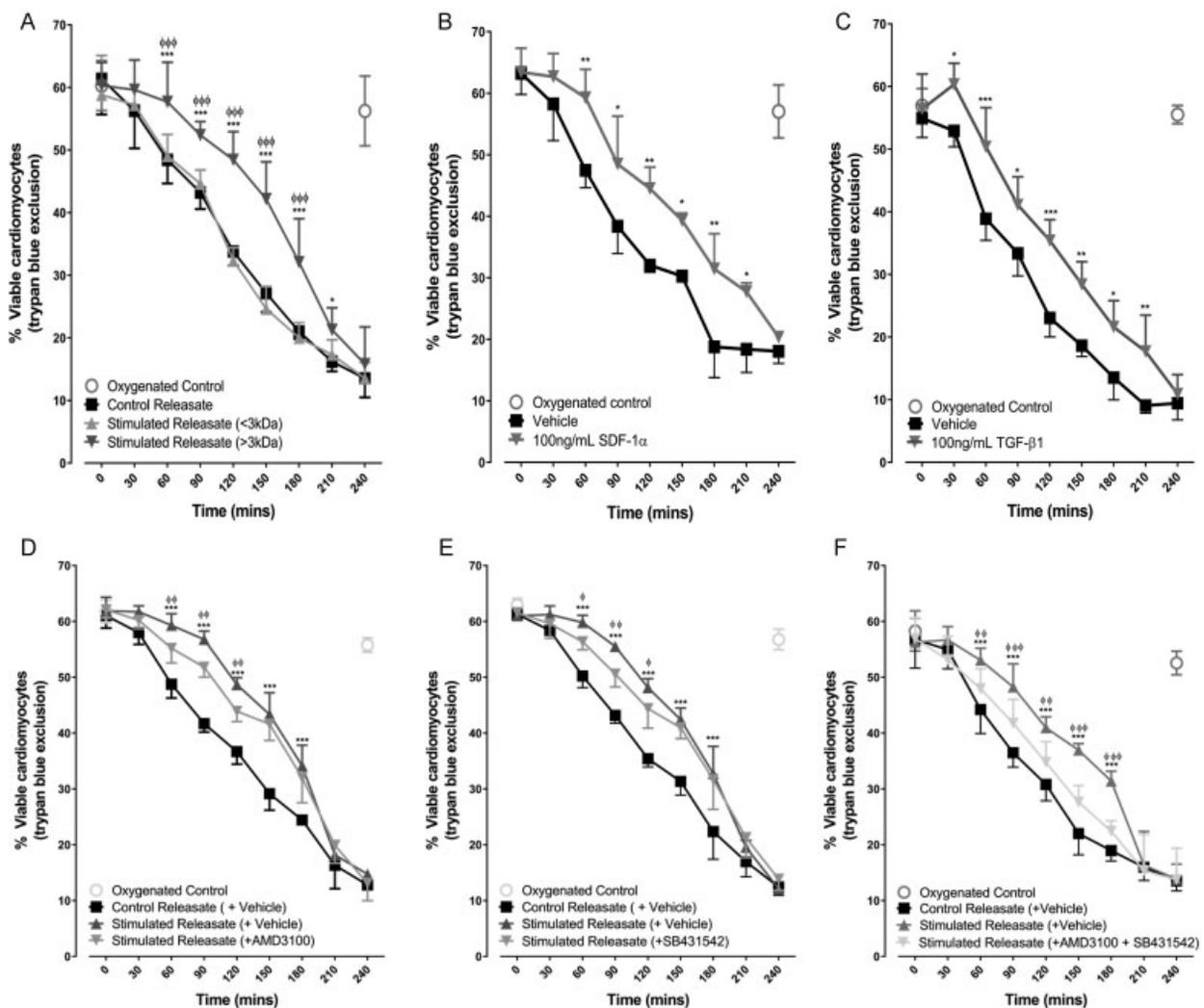


Fig. 2 Role for platelet α -granule proteins, SDF-1 α and TGF- β 1, in mediating the protective effects of platelet secreted factors. (A–F) Cardiomyocytes were preconditioned with releasates from vehicle-treated (control) WT platelets: (A) filtered, <3 kDa and retained, >3 kDa releasates from CRP-treated (stimulated) WT platelets; (B) recombinant SDF-1 α (100 ng/mL); (C) recombinant TGF- β 1 (100 ng/mL) and (D–F) unfiltered releasate from CRP-treated (stimulated) WT platelets, then monitored for cardiomyocyte viability for up to 240 minutes of ischemic injury, with nonischemic (oxygenated control) samples monitored at 0- and 240-minute time points. (D–F) Cardiomyocytes were pretreated with vehicle control (0.1% DMSO) or inhibitors: (D) 0.5 μ M AMD3100 (CXCR4 antagonist); (E) 1 μ M SB431542 (TGFBR1 antagonist), and (F) 0.5 μ M AMD3100 and 1 μ M SB431542 for 30 minutes prior to releasate preconditioning. Data are mean \pm SD ($n = 4$ for (A) and (F), $n = 3$ for B–E); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control releasate (A, D–F)/vehicle (B,C) and $\phi p < 0.05$, $\phi\phi p < 0.01$, $\phi\phi\phi p < 0.001$ versus stimulated releasate (<3 kDa (A)) / + AMD3100 (D)) / + SB431542 (E)/AMD3100 + SB431542 (F)).

Cardioprotective Roles for Platelet α -Granule Proteins SDF-1 α and TGF- β 1

Platelet α -granules are loaded with functionally active proteins, some of which have been previously implicated as having protective effects on cardiac tissue, including the cytokines stromal cell-derived factor (SDF)-1 α and transforming growth factor (TGF)- β 1.^{20,21} Initially, we demonstrated that recombinant SDF-1 α or TGF- β 1 could significantly delay cardiomyocyte death during ischemia compared with vehicle-treated cardiomyocytes (**Fig. 2B, C**). Subsequently, we assessed the functional relevance of platelet-derived SDF-1 α and TGF- β 1 by targeting their respective receptors, CXCR4 and TGF β R1, both of which are expressed on cardiomyocytes.^{22,23} Blocking CXCR4 with AMD3100 or TGF β R1 with SB431542 on cardiomyocytes caused a subtle, yet significant reversal of the cardioprotective effect of the stimulated platelet releasate between 60 and 120 minutes of ischemia (**Fig. 2D, E**). However, blocking both receptors simultaneously caused a more profound attenuation of the protective effect and persisted from 60 up to 180 minutes of ischemia, at which point the protective effect typically dissipates (**Fig. 2F**). Control experiments with AMD3100 or SB431542 did not reveal any baseline effects on cardiomyocyte viability during ischemia when compared with vehicle-treated cardiomyocytes (data not shown).

Protein Kinase C Is Essential for Platelet Releasate-Mediated Cardioprotection

Considering the protective effects of the stimulated platelet releasates, we sought to determine possible prosurvival

signaling mechanisms activated within cardiomyocytes during ischemia. By measuring serine phosphorylated PKC substrates, we observed that stimulated platelet releasates enhanced PKC activity between 30 and 120 minutes of ischemia relative to control releasate-treated cardiomyocytes (**Fig. 3A**). However, we did not detect any temporal differences in the phosphorylation kinetics of the kinases, Akt or Erk 1/2 between treatments (**Fig. 3A**). To assess a potentially causal role for PKC, pretreatment of cardiomyocytes with a pan PKC inhibitor, BIM I, completely suppressed the cardioprotective response of the stimulated platelet releasate during ischemia (**Fig. 3B**). Control experiments with BIM I alone treated cardiomyocytes confirmed that PKC inhibition does not induce a baseline reduction in cardiomyocyte viability during ischemia, relative to vehicle-treated cardiomyocytes (**Fig. S4**, supplementary figure available in the online version only).

Clinical Antiplatelet Target P2Y₁₂, but Not cyclooxygenase-1, Is Important for Cardioprotective Effects of Platelets

Clinically, a mainstay of therapy for myocardial infarction is the rapid control of platelet function to prevent further thrombotic and ischemic episodes and to help destabilize any ongoing thrombotic event. Antiplatelet therapies target the activatory adenosine diphosphate (ADP) and thromboxane A₂ amplification pathways in platelets by blocking the P2Y₁₂ receptor, via clopidogrel and cyclooxygenase (COX)-1, via low-dose aspirin, respectively. Under our conditions,

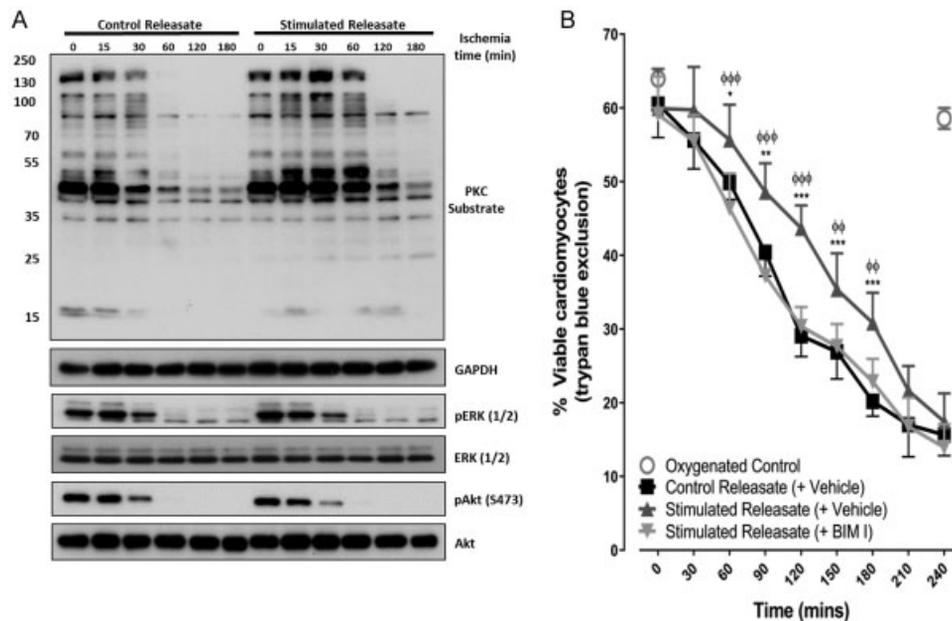


Fig. 3 Protein kinase C (PKC) activity is essential for platelet releasate-mediated cardioprotection. (A) Cardiomyocytes preconditioned with releasates from vehicle- (control) and CRP- (stimulated) treated WT platelets were subjected to ischemic injury, with samples removed from the pellet and lysed at indicated ischemic time points. Samples were monitored for changes in PKC activity (Phospho serine PKC substrate), phospho Akt (Ser473), and Erk 1/2 (Thr202/Tyr204) by immunoblotting with specific antibodies followed by appropriate loading controls. Blots shown are representative of three independent experiments. (B) Cardiomyocytes were pretreated in the absence (+ DMSO vehicle) or presence of 10 μ M BIM I for 30 minutes prior to preconditioning with releasates from vehicle- (control) and CRP-treated (stimulated) WT platelets, then monitored for cardiomyocyte viability for up to 240 minutes of ischemic injury, with nonischemic (oxygenated control) samples monitored at 0- and 240-minute time points. Data are mean \pm SD; $n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control releasate, $^{\Phi}p < 0.01$, $^{\Phi\Phi}p < 0.001$ versus stimulated releasate (+ BIM I). Two-way ANOVA with Bonferroni post hoc test.

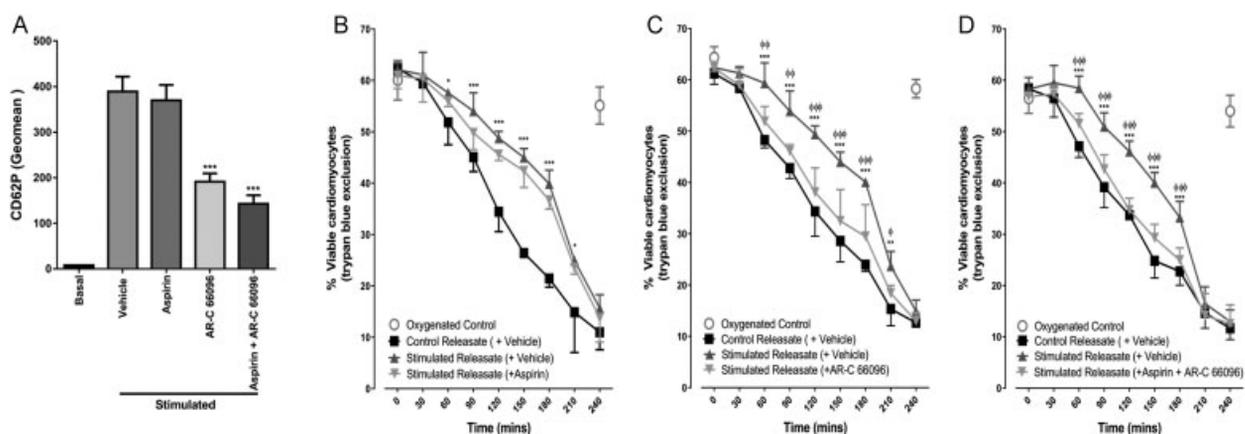


Fig. 4 Targeting platelet P2Y₁₂, but not cyclooxygenase, reduces platelet secretion and cardioprotection. (A) Washed platelets (2×10^8 /mL) from WT mice were pretreated with vehicle (Tyrode's) or inhibitors; 30 μ M aspirin (cyclooxygenase) and 1 μ M AR-C 66096 (P2Y₁₂ antagonist) for 10 minutes, then stimulated with 10 μ g/mL CRP and analyzed for α -granule secretion by surface CD62P exposure. Data are mean \pm SD; $n = 4$, $***p < 0.001$, one-way ANOVA with Bonferroni post hoc test. (B–D) Cardiomyocytes preconditioned with releasates from vehicle-treated (control) or CRP-treated (stimulated) WT platelets in the absence (+ Tyrode's vehicle) or presence of (B) 30 μ M aspirin, (C) 1 μ M AR-C 66096, and (D) 30 μ M aspirin + 1 μ M AR-C 66096 were monitored for changes in viability during ischemic injury, with nonischemic (oxygenated control) samples monitored at 0 and 240 minutes time points. Data are mean \pm SD; $n = 4$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus WT-controlled releasate (+ vehicle (B–D)) and $^{\oplus}p < 0.05$, $^{\oplus\oplus}p < 0.01$, $^{\oplus\oplus\oplus}p < 0.001$ versus stimulated releasate (+Aspirin (B)/ + AR-C66096 (C)/ + Aspirin + AR-C66096 (D)), two-way ANOVA with Bonferroni post hoc test.

pretreating platelets with the P2Y₁₂ inhibitor, AR-C 66096, reduced CRP-induced α -granule secretion by approximately 51%, whereas aspirin had no significant effect (\rightarrow Fig. 4A). Consistent with the effect on α -granule secretion, blockade of P2Y₁₂ in platelets, alone or in combination with aspirin, substantially attenuated the subsequent protective effect of the stimulated releasate on cardiomyocyte viability during ischemia (\rightarrow Fig. 4C, D), whereas stimulated releasates from aspirin alone pretreated platelets did not exhibit a significant reduction in cardioprotection compared with stimulated releasates from vehicle-treated platelets (\rightarrow Fig. 4B).

Discussion

Platelets play a critical role in the pathogenesis of myocardial infarction, initiating the ischemic damage through the thrombotic plug that they form in the coronary vessels. As part of their activity, however, platelets secrete cargo that is able to exert localized effects on numerous cells at the injury site, including leukocytes and endothelial cells.^{24,25} Here, we hypothesized that through secretion of multiple bioactive mediators platelets may play a paradoxical protective role in addition to their induction of ischemic damage. This study reveals new paracrine signaling capabilities of platelets to modulate the sensitivity of myocardial cardiomyocytes to ischemic injury.

It is well described that the myocardium possesses its own inherent, cardioprotective capacity as short bouts of ischemia, followed by reperfusion, and can protect cardiomyocytes from prolonged exposure to ischemia, a phenomenon referred to as ischemic preconditioning (IPC).²⁶ Importantly, the isolated in vitro model used in this study, which was first described by Vander Heide et al, has been effectively utilized to recapture the IPC effect observed from various in vivo and

ex vivo models.^{16,27} Our observations that released cargo from platelets stimulated with maximal doses of CRP could temporally delay the kinetics of cardiomyocyte death are consistent with the responses observed from earlier studies demonstrating the protective effects of acute IPC.^{27,28} Inevitably, absolute protection of cardiomyocytes is not sustained with prolonged ischemia and this acute protective effect is lost by 240 minutes of ischemia. Importantly, the receptor for CRP, GPVI, has established roles in platelet-mediated coronary thrombosis, but is also platelet-specific, allowing for discrimination against any potential "off-target" effects on cardiomyocytes that other common platelet agonists such as thrombin, ADP, and thromboxane A₂ may incur.

To explore the most probable candidate sources of biomolecules within the platelet releasate, we utilized platelets from *Unc13d^{flⁱⁿx}* mice, which are unable to secrete dense granule cargo and have severely compromised α -granule (\rightarrow Fig. 1C) and lysosomal cargo release.²⁹ Notably, CRP-stimulated platelet releasates from these mice completely lost the protective effect on cardiomyocyte viability during ischemia compared with WT platelets. Dense granules are loaded with high concentrations of low-molecular-weight components, in particular adenosine nucleotides (ADP and ATP), divalent cations (Ca²⁺, Mg²⁺), and neurotransmitters (serotonin, histamine) and are critical regulators of thrombosis and vascular remodeling.³⁰ Separating these components from larger α -granule cargo by fractionation showed that the dense granule content was incapable of supporting cardioprotection. Interestingly, adenosine has previously been shown to have a cardioprotective effect and could be formed by degradation of secreted ADP and ATP by platelet surface ectonucleotidases, CD39 and CD73.³¹ However, preconditioning cardiomyocytes with adenosine did not significantly delay the rate of cardiomyocyte death, providing

further supportive evidence for dense granule cargo lacking a cardioprotective role in our model (►Fig. S2B, supplementary figure available in the online version only).

Platelet lysosomal number is low and their cargo, which contains numerous proteases implicated in vessel remodeling post injury, makes it an unlikely source of cardioprotective factors. Conversely, platelet α -granules, of which there are approximately 50 to 80 per platelet, are loaded with functionally active cargo with the ability to target numerous vascular cells.³² Pertinent to our findings, the α -granule proteins, SDF-1 α and TGF- β 1, both of which are known to be secreted from activated platelets,³³ have also been implicated in cardioprotection. We observed that pretreating cardiomyocytes with recombinant SDF-1 α or TGF- β 1 could substantially delay the rate of cardiomyocyte death during ischemia, in a manner similar to stimulated platelet releasates, but individually blocking the respective receptors for these ligands, CXCR4 and TGF β 1R1, could only partially block the cardioprotective effect of the releasate. Importantly, the dose of the receptor antagonist was sufficient to completely block the effect of their respective recombinant ligand (►Fig. S3A, B, supplementary figure available in the online version only). The apparent discrepancy between findings with the platelet releasate and recombinant pretreatments likely reflects differences in soluble ligand concentration between the two, but may also reflect the opposing, functional nature of different components of the complex secreted platelet cargo.³⁴ Interestingly, simultaneous blockade of CXCR4 and TGF β 1R1 caused a more substantial attenuation in platelet releasate-mediated cardioprotection, suggesting a synergistic or additive contribution of different cargo to the protective effect. However, the attenuation was not complete, implicating other secreted platelet granule contents, possibly additional α -granule peptide(s), as additive or synergistic regulators in this complex releasate. Indeed, synergistic behavior of various growth factors toward scavenger receptor activity in smooth muscle cells has been previously reported and we speculate that a similar modality of function may be in effect here.³⁵

Mechanistically, we wanted to explore possible pro-survival targets within cardiomyocytes that were sensitive to releasate stimulation. The kinases Akt and Erk1/2 have been previously linked to cardioprotection, but we did not detect any temporal differences in phosphorylation kinetics between vehicle- and CRP-stimulated platelet releasates during ischemia.^{36,37} However, we did detect prolonged PKC activity during ischemia in the stimulated releasate samples. Pertinent to this, the PKC family is widely recognized for its regulatory roles in IPC,^{38,39} and previous studies have also identified signaling links between either SDF-1 α or TGF- β 1 and PKC.^{40,41} Findings from our functional ischemia assay suggest that PKC signaling within cardiomyocytes is essential to the protective effect of the releasate. The end effectors that functionally couple PKC activity to a cardioprotective response are still not determined. Some evidence suggests that PKC activity increases opening of the mitochondrial and sarcolemmal ATP-dependent potassium (K_{ATP}) channels, which lowers Ca^{2+} loading during ischemia and

protects the mitochondrial permeability transition pore (mPTP) from the deleterious effects of elevated Ca^{2+} . Other studies, however, dispute the K_{ATP} channel theory, suggesting that a lowering of oxidative stress during ischemia desensitizes the mPTP to the deleterious effects of Ca^{2+} loading.^{42,43}

Undoubtedly, the initiating predisposition to myocardial ischemic injury is a platelet-mediated process and antiplatelet therapies are prophylactically administered as effective tools in the primary and secondary prevention of occlusive thrombosis.⁴⁴ Interestingly, blockade of P2Y₁₂, but not aspirin, significantly reduced the cardioprotective effect of the platelet releasate. Unlike stimulated releasates from Munc13-4^{-/-} platelets, the reversal of the protective effect with P2Y₁₂ inhibition was not complete and likely reflects the greater attenuation of platelet α -granule secretion in *Unc13d^{flin}* mice. Consistently, aspirin treatment did not significantly alter α -granule secretion or cardioprotection from the platelet releasate. Findings from in vivo studies of myocardial ischemia-reperfusion (I/R) injury, by coronary artery ligation, may seem to contradict our data, as different studies have demonstrated cardioprotective effects of the antiplatelet therapies and also implicated platelet-specific roles during I/R injury pathogenesis.^{10,45,46} Interestingly, other studies have demonstrated that the P2Y₁₂ antagonist, clopidogrel, had no effect on myocardial I/R injury, despite its antiplatelet effect, while it has also been suggested that the cardioprotective effect of a more recently developed P2Y₁₂ antagonist, ticagrelor, may be independent of its antiplatelet properties.^{47,48} Notably, the mechanical nature of ligation in these in vivo models, in place of occlusive thrombosis due to endothelial disruption, raises issues concerning the mechanism of platelet activation and secretion, and makes accurate inferences more challenging. The in vitro model used here is also not without limitations, as it lacks the mechanics of reperfusion hypercontracture from an intact myocardium and also the influence of other vascular cells.⁴⁹ It does also presume that soluble platelet cargo can target the myocardium during thrombosis. However, in light of evidence demonstrating a functional crosstalk between endothelial growth factors and cardiomyocytes, it is probable that such a conduit exists during endothelial disruption and coronary thrombosis.¹⁴ Importantly, the model does allow us to mimic platelet activation and secretion relevant to thrombosis, which is currently lacking in the coronary ligation model of myocardial infarction. Our approach therefore allowed us to monitor cardiomyocyte viability over time during ischemia, and the effect of activated platelet releasates on this viability.

Overall, our findings suggest that platelet-released factors enhance a therapeutic window of cardiomyocyte survival during ischemia, highlighting a double-edged sword functionality of platelets because they are also causative of the initial ischemic insult. This adds to the expanding list of nonclassical roles for platelets, and also highlights important considerations for antiplatelet therapies that alter platelet activity and in particular granule secretion, as this cargo possesses numerous beneficial influences, for example, in

the context of wound healing,⁵⁰ yet deleterious consequences in the context of tumor growth and stability.⁵ The intriguing observation in this study is that in the context of ischemic damage to cardiomyocytes, platelet P2Y₁₂, but not cyclooxygenase, blockade may block the release of protective components from platelets. The supposition here is not to undermine the protective capabilities of P2Y₁₂ inhibitors in the primary prevention of ischemic tissue damage, but to raise important issues regarding the utility of antiplatelet therapies that impact on nonclassical aspects of platelet biology.

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Conflict of Interest

None declared.

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References

- Rafii S, Cao Z, Lis R, et al. Platelet-derived SDF-1 primes the pulmonary capillary vascular niche to drive lung alveolar regeneration. *Nat Cell Biol* 2015;17(02):123–136
- Lynch SE, Nixon JC, Colvin RB, Antoniades HN. Role of platelet-derived growth factor in wound healing: synergistic effects with other growth factors. *Proc Natl Acad Sci U S A* 1987;84(21):7696–7700
- Xiang B, Zhang G, Guo L, et al. Platelets protect from septic shock by inhibiting macrophage-dependent inflammation via the cyclooxygenase 1 signalling pathway. *Nat Commun* 2013;4:2657
- Jørgensen L. The role of platelets in the initial stages of atherosclerosis. *J Thromb Haemost* 2006;4(07):1443–1449
- Ho-Tin-Noé B, Goerge T, Cifuni SM, Duerschmied D, Wagner DD. Platelet granule secretion continuously prevents intratumor hemorrhage. *Cancer Res* 2008;68(16):6851–6858
- Nieswandt B, Hafner M, Echtenacher B, Männel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. *Cancer Res* 1999;59(06):1295–1300
- Coppinger JA, Cagney G, Toomey S, et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* 2004;103(06):2096–2104
- Golebiewska EM, Poole AW. Secrets of platelet exocytosis - what do we really know about platelet secretion mechanisms? *Br J Haematol* 2013;165(02):204–216
- Walsh TG, Metharom P, Berndt MC. The functional role of platelets in the regulation of angiogenesis. *Platelets* 2015;26(03):199–211
- Pachel C, Mathes D, Arias-Loza AP, et al. Inhibition of platelet glycoprotein VI protects against myocardial ischemia-reperfusion injury. *Arterioscler Thromb Vasc Biol* 2016;36(04):629–635
- Devanathan V, Hagedorn I, Köhler D, et al. Platelet Gi protein Gα₂ is an essential mediator of thrombo-inflammatory organ damage in mice. *Proc Natl Acad Sci U S A* 2015;112(20):6491–6496
- Yang BC, Mehta JL. Platelet-derived adenosine contributes to the cardioprotective effects of platelets against ischemia-reperfusion injury in isolated rat heart. *J Cardiovasc Pharmacol* 1994;24(05):779–785
- Yang BC, Virmani R, Nichols WW, Mehta JL. Platelets protect against myocardial dysfunction and injury induced by ischemia and reperfusion in isolated rat hearts. *Circ Res* 1993;72(06):1181–1190
- Parodi EM, Kuhn B. Signalling between microvascular endothelium and cardiomyocytes through neuregulin. *Cardiovasc Res* 2014;102(02):194–204
- Harper MT, van den Bosch MT, Hers I, Poole AW. Absence of platelet phenotype in mice lacking the motor protein myosin Va. *PLoS One* 2013;8(01):e53239
- Vander Heide RS, Rim D, Hohl CM, Ganote CE. An in vitro model of myocardial ischemia utilizing isolated adult rat myocytes. *J Mol Cell Cardiol* 1990;22(02):165–181
- Wang F, He Q, Sun Y, Dai X, Yang XP. Female adult mouse cardiomyocytes are protected against oxidative stress. *Hypertension* 2010;55(05):1172–1178
- Gawaz M. Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovasc Res* 2004;61(03):498–511
- Savage JS, Williams CM, Konopatskaya O, Hers I, Harper MT, Poole AW. Munc13-4 is critical for thrombosis through regulating release of ADP from platelets. *J Thromb Haemost* 2013;11(04):771–775
- Hu X, Dai S, Wu WJ, et al. Stromal cell derived factor-1 alpha confers protection against myocardial ischemia/reperfusion injury: role of the cardiac stromal cell derived factor-1 alpha CXCR4 axis. *Circulation* 2007;116(06):654–663
- Yang BC, Zander DS, Mehta JL. Hypoxia-reoxygenation-induced apoptosis in cultured adult rat myocytes and the protective effect of platelets and transforming growth factor-beta(1). *J Pharmacol Exp Ther* 1999;291(02):733–738
- Döring Y, Pawig L, Weber C, Noels H. The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease. *Front Physiol* 2014;5:212
- Koitabashi N, Danner T, Zaiman AL, et al. Pivotal role of cardiomyocyte TGF-β signaling in the murine pathological response to sustained pressure overload. *J Clin Invest* 2011;121(06):2301–2312
- Gardiner EE, De Luca M, McNally T, Michelson AD, Andrews RK, Berndt MC. Regulation of P-selectin binding to the neutrophil P-selectin counter-receptor P-selectin glycoprotein ligand-1 by neutrophil elastase and cathepsin G. *Blood* 2001;98(05):1440–1447
- Henn V, Slupsky JR, Gräfe M, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;391(6667):591–594
- Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74(05):1124–1136
- Armstrong S, Ganote CE. Preconditioning of isolated rabbit cardiomyocytes: effects of glycolytic blockade, phorbol esters, and ischaemia. *Cardiovasc Res* 1994;28(11):1700–1706
- Armstrong SC, Kao R, Gao W, et al. Comparison of in vitro preconditioning responses of isolated pig and rabbit cardiomyocytes: effects of a protein phosphatase inhibitor, fostriecin. *J Mol Cell Cardiol* 1997;29(11):3009–3024
- Ren Q, Wimmer C, Chicka MC, et al. Munc13-4 is a limiting factor in the pathway required for platelet granule release and hemostasis. *Blood* 2010;116(06):869–877

- 30 King SM, McNamee RA, Houg AK, Patel R, Brands M, Reed GL. Platelet dense-granule secretion plays a critical role in thrombosis and subsequent vascular remodeling in atherosclerotic mice. *Circulation* 2009;120(09):785–791
- 31 Colgan SP, Eltzschig HK, Eckle T, Thompson LF. Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal* 2006;2(02):351–360
- 32 Heijnen H, van der Sluijs P. Platelet secretory behaviour: as diverse as the granules ... or not? *J Thromb Haemost* 2015;13(12):2141–2151
- 33 Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. *Blood* 2012;120(26):5209–5216
- 34 Italiano JE Jr, Richardson JL, Patel-Hett S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood* 2008;111(03):1227–1233
- 35 Gong Q, Pitas RE. Synergistic effects of growth factors on the regulation of smooth muscle cell scavenger receptor activity. *J Biol Chem* 1995;270(37):21672–21678
- 36 Fryer RM, Pratt PF, Hsu AK, Gross GJ. Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. *J Pharmacol Exp Ther* 2001;296(02):642–649
- 37 Kunuthur SP, Mocanu MM, Hemmings BA, Hausenloy DJ, Yellon DM. The Akt1 isoform is an essential mediator of ischaemic preconditioning. *J Cell Mol Med* 2012;16(08):1739–1749
- 38 Fryer RM, Schultz JE, Hsu AK, Gross GJ. Importance of PKC and tyrosine kinase in single or multiple cycles of preconditioning in rat hearts. *Am J Physiol* 1999;276(4, Pt 2):H1229–H1235
- 39 Ytrehus K, Liu Y, Downey JM. Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol* 1994;266(3, Pt 2):H1145–H1152
- 40 Lim JY, Park SJ, Hwang HY, et al. TGF-beta1 induces cardiac hypertrophic responses via PKC-dependent ATF-2 activation. *J Mol Cell Cardiol* 2005;39(04):627–636
- 41 Odemis V, Boosmann K, Dieterlen MT, Engele J. The chemokine SDF1 controls multiple steps of myogenesis through atypical PKCzeta. *J Cell Sci* 2007;120(Pt 22):4050–4059
- 42 Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta* 2007;1767(08):1007–1031
- 43 Suzuki M, Sasaki N, Miki T, et al. Role of sarcolemmal K(ATP) channels in cardioprotection against ischemia/reperfusion injury in mice. *J Clin Invest* 2002;109(04):509–516
- 44 Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov* 2010;9(02):154–169
- 45 Fu Y, Wang Z, Chen WL, Moore PK, Zhu YZ. Cardioprotective effects of nitric oxide-aspirin in myocardial ischemia-reperused rats. *Am J Physiol Heart Circ Physiol* 2007;293(03):H1545–H1552
- 46 Bell RM, Sivaraman V, Kunuthur SP, Cohen MV, Downey JM, Yellon DM. Cardioprotective properties of the platelet P2Y12 receptor inhibitor, Cangrelor: protective in diabetics and reliant upon the presence of blood. *Cardiovasc Drugs Ther* 2015;29(05):415–418
- 47 Ye Y, Birnbaum GD, Perez-Polo JR, Nanhwan MK, Nylander S, Birnbaum Y. Ticagrelor protects the heart against reperfusion injury and improves remodeling after myocardial infarction. *Arterioscler Thromb Vasc Biol* 2015;35(08):1805–1814
- 48 Nanhwan MK, Ling S, Kodakandla M, Nylander S, Ye Y, Birnbaum Y. Chronic treatment with ticagrelor limits myocardial infarct size: an adenosine and cyclooxygenase-2-dependent effect. *Arterioscler Thromb Vasc Biol* 2014;34(09):2078–2085
- 49 Diaz RJ, Wilson GJ. Studying ischemic preconditioning in isolated cardiomyocyte models. *Cardiovasc Res* 2006;70(02):286–296
- 50 Ma L, Elliott SN, Cirino G, Buret A, Ignarro LJ, Wallace JL. Platelets modulate gastric ulcer healing: role of endostatin and vascular endothelial growth factor release. *Proc Natl Acad Sci U S A* 2001;98(11):6470–6475