Trapped Platelets Activated in Ischemia Initiate Ventricular Fibrillation

Running title: Dhanjal et al.; platelets and ventricular fibrillation

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Journal Subject Codes: [132] Arrhythmias - basic studies, [130] Animal models of human disease, [151] Ischemic biology - basic studies
Abstract:

Background - We tested the hypothesis that ischemia-induced ventricular fibrillation (VF) is facilitated by platelets, trapped regionally in the ischemic zone (IZ) and activated to release arrhythmogenic secretome.

Methods and Results - In a randomized study, in rat blood-free buffer-perfused isolated hearts, IZ territory (34 ± 1% of LV) was selected so that ischemia evoked VF in only 42% of controls. VF incidence was increased to 91% (P<0.05) by coronary ligation-induced trapping of freshly-prepared autologous platelets (infused before and during coronary ligation, with trapping confirmed by 111In-labeled platelet autoradiographic imaging). Trapping of platelet secretome prepared ex vivo, or platelet sized fluorospheres, did not increase ischemia-induced VF incidence. Secretome alone did however evoke VF in two sham coronary-ligated hearts. Perfusion did not activate infused platelets in sham coronary-ligated hearts, whereas ligation activated trapped platelets (assessed by thromboxane release). In a separate study, trapping whole heparinised blood mimicked the ability of trapped platelets to increase VF incidence. This effect was not prevented by 5+day oral pre-treatment in vivo with clopidogrel (10 mg/kg/day) or indomethacin (2.4 mg/kg/day).

Conclusions - Platelets facilitate VF during acute ischemia independently of their ability to participate in occlusive thrombosis. Moreover the effect is unresponsive to commonly used antiplatelet drugs. Labile secretome constituents appear to be responsible. This opens a novel avenue for antiarrhythmic drug research.

Key words: arrhythmia (mechanisms), platelet, myocardial ischemia, antiarrhythmia agent, sudden cardiac death, arrhythmia, antiarrhythmic drug development
Introduction

VF, the principle cause of sudden cardiac death (SCD), is largely resistant to ion channel-targeting antiarrhythmic drugs. The pathophysiology of ischemia (that mediates the adverse electrophysiological milieu) may represent a more promising target.

Animal studies have shown that early (phase 1) VF arises during the first 30 minutes of ischemia when arrhythmogenic substances accumulate, but it remains unclear which substances are sufficient and necessary mediators of VF.

Mediators may derive from ischemic myocytes, and from other local tissue (including platelets). Thus platelets may facilitate VF independently of their role in thrombus formation. Clinical trials suggest the mortality benefit of anti-platelet agents such as aspirin and αIIbβ3 integrin antagonists may result from effects more complex than simple thrombus inhibition.

In animal studies, several platelet activating factor (PAF) antagonists reduced ischemia-induced VF incidence. Moreover, aspirin pretreatment reduced ischemia-induced VF in various animal models. However, there is a lack of evidence directly linking anti-VF effects to platelet ablation. It has been argued that a systematic examination of the role of platelets in VF initiation during ischemia is long overdue.

We have tested whether VF is facilitated by the activation of platelets trapped within the ischemic territory resulting in the release of pro-arrhythmic secretome. A novel in vitro model system permitted myocardial ischemia to be induced in isolated rat hearts in the absence or presence of an autologous infusion of platelets. When IZ was made deliberately suboptimal, platelets facilitated the ability of ischemia to evoke VF, and secretome evoked VF in the absence of ischemia. Facilitation of VF was not attenuated by current clinical anti-platelet therapy.
Methods

Animals and core methods

Experiments were performed according to the United Kingdom Home Office Guide on the Operation of the Animals (Scientific Procedures) Act 1986. Heart perfusion, induction of ischemia, verification and quantification of IZ size, and data recording and analysis were as described previously. Male Wistar rats (220-270 g) were anaesthetised (pentobarbitone 60 mg/kg i.p.) and given sodium heparin 250 i.u., i.p. Blood samples (7 – 10 mL) for platelet preparation were drawn by inferior vena caval puncture and collected into acid-citrate-dextrose buffer (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid). Rats were then killed by cardiac excision, and hearts excised and immersed in cold (4 °C) Krebs solution (modified to contain 3 meq/l K+ and 1.4 meq/l Ca++) then cannulated for perfusion with similar Krebs solution (gased with 95% O2 + 5% CO2) at 37 °C at a constant pressure (70 mmHg). A silver electrogram (ECG) lead was positioned to impale a part of the anterior surface of the left ventricle that, from experience, would be subserved by the artery to be occluded. The ECG was recorded (PowerLab, ADInstruments, UK) at a sampling rate of 2 kHz and with high- and low-pass frequency filters set at 0.3 Hz and 1 kHz. The standard definitions of rat ECG intervals were used. Experimental guiding principles and arrhythmia definitions were as described in the Lambeth Conventions. Coronary flow (CF) was measured by timed collection of coronary effluent. A silk suture (3/0 thread), sewn under the left main artery immediately after set-up, was tightened to achieve regional ischemia and the size of the IZ was determined at the end of perfusion using the disulphine blue method.

Platelet preparation and control groups

Washed platelets were derived by centrifugation, and re-suspended (2x10^8/ml) in Krebs-solution...
then rested for at least 10 minutes prior to use. To validate platelet viability and aggregability when suspended in Krebs solution (the preparation method is validated only with Tyrode solution), platelet aggregometry was performed\textsuperscript{22} using fibrillar-type I horn collagen (Nycomed, Axis-Shield, UK), thrombin, (Sigma, Poole, UK) and ADP (Sigma, Poole, UK).

Three control groups were used in the first of two studies: platelet vehicle control solution (Krebs), fluorospheres, and secretome. The fluorosphere control group tested whether any increase in arrhythmias in the platelet group was mediated by the mere presence of platelet sized particles. The fluorospheres (Invitrogen, Paisley, UK) mimic platelet size and charge. Platelet secretome controls tested whether secretome facilitates ischemia-induced VF and whether secretome can evoke VF in the absence of ischemia. Secretome contains substances (e.g., ATP, serotonin, histamine and more than 300 proteins),\textsuperscript{23, 24} many of which can alter cardiac electrophysiology.\textsuperscript{24} To obtain secretome, the washed platelet pellet was resuspended in 4 ml Krebs solution and stimulated with 1 U/ml thrombin. The aggregated suspension was then centrifuged (1000g for 3 minutes) and the secretome collected.

Platelets, fluorospheres and secretome were prepared in 4 ml of warmed gased Krebs solution immediately before infusion, and contained $2 \times 10^8$/ml platelets, $4 \times 10^9$/ml fluorospheres or (as quantified by Bradford assay) 0.21mg/mL protein, respectively.

**Platelet delivery system**

A Langendorff cannula was modified to allow infusion of washed platelets into the perfusate in a non-activated and functional state (Figure 1). Platelets, platelet vehicle, fluorospheres or secretome were delivered as ‘infusion solutions’ to the cannula from polypropylene syringes attached to a syringe driver.
Experimental design

In the first study, platelets were trapped in the IZ by coronary ligation. To validate the method, hearts were perfused with platelets radiolabelled with 50MBq 111In oxine sulphate. After coronary ligation hearts were frozen in liquid nitrogen for autoradiography performed on 20 micron sections exposed to CL-XPosure Film (Thermo Fisher Scientific, Loughborough, UK) for 3 days. Then 96 rats were randomised into 8 groups: Krebs solution controls, washed platelets, platelet secretome or fluorospheres, each with coronary ligation or sham ligation. All hearts were perfused for 30 minutes with Krebs solution, then infusion solution was delivered at 0.2 ml/min for 20 min, with coronary ligation (or sham ligation) performed at the half way point (10 min).

Thromboxane assay

In separate groups of hearts, at the end of platelet infusion (after 10 min ischemia) the ligature was released and coronary effluent collected for 60 s. Thromboxane (TX) B2 was measured by ELISA assay (Biotrak thromboxane B2 enzymeimmunoassay system, GE Healthcare, Amersham, UK). The TX measured is a marker of platelet activation and degranulation during ischemia, and is not ‘reperfusion-induced’ release, since true reperfusion-induced platelet adhesion and activation take several minutes to manifest.

Drug administration and blood trapping

In a second study, an equivalent protocol was used to explore the ability of whole blood to mimic the ability of trapped platelets to facilitate ischemia-induced VF, and test the effects of standard ‘antiplatelet’ drugs. A blood sample was taken prior to cardiac excision, heparinised (0.1 U/ml), then delivered via the system used earlier (4 ml over 20 min at a rate of rate 0.2 ml/min) with coronary ligation and blood trapping performed half way through the infusion. Rats received p.o.
indomethacin (n=14; 2.4 mg/kg/day) (Sigma, Poole, UK), clopidogrel (n=9; 10 mg/kg/day) (Wockhardt, Wrexham, UK), or vehicle (n=20) (1ml/kg/day of 4% v/v ethanol in distilled water) for a minimum of 5 consecutive days. The vehicle group was subdivided to provide blood-trapped (n=8) and blood-free (Krebs solution throughout, n=12) controls.

**Exclusion criteria**

Based on previously published standards, hearts were excluded if pre-ligation sinus heart rate (HR) was <200 beats per minute, or CF was <5 mL/min\(^2\) or if IZ was outside the range of 25-40% of total ventricular weight, or VF occurred before the onset of ischemia. VF susceptibility in individual hearts is not dependent on baseline HR or CF beyond these stated exclusion limits\(^2\). Blood-perfused hearts were excluded if they developed an intracoronary thrombus prior to coronary ligation. The studies required 204 rats of which 12 were excluded.

**Statistical Analyses**

Parametric variables (+SEM) including CF, HR, PR interval, QT interval at 90% repolarization (QT90)\(^2\) and light transmission for platelet aggregation were evaluated by one-way analysis of variance (ANOVA) and Dunnett’s test to compare ‘treatments’ with control. Incidences of VT and VF (dichotomous variables) were analysed by Fisher’s exact test due to small sample sizes. This test was used for global multiple comparisons and if significant, a pairwise comparison was performed versus controls using the same test. Other non-parametric variables were analysed using the Steel Dwass test, and the Kruskal Wallis test was used for the estimation of stochastic probability in intergroup comparisons. P-values are two-sided and results were deemed statistically-significant when p < 0.05. Statistical analyses were performed on R version 2.14.
Results

Delivered platelets were viable and functional

Aggregation traces demonstrated normal dose response curves to agonists (Figure 2A-C), and 1 UmL⁻¹ of thrombin induced a maximal aggregation response (Figure 2D). Washed platelets were non-activated on exiting the delivery system set up without a heart attached, but could be fully activated by agonist stimulation (Figure 2E), validating the platelet delivery system, and Krebs solution as a platelet vehicle.

Platelet trapping facilitates ischemia-induced VF

Coronary ligation trapped infused platelets within the IZ (Figure 3). Mean IZ values (33-35%) were suboptimal for VF (Table 1) meaning that ischemia-induced VF occurred in only 42% of platelet-free controls as desired. ²⁸ Platelet trapping increased VF incidence to 91% (P<0.05) whereas trapping of secretome or fluorospheres had no such effect (Figure 4). Delivery of platelets in the absence of ischemia did not elicit VF (either in shams, or pre-ischemia in hearts with coronary ligation). However, two sham coronary-ligated hearts experienced VF during secretome infusion.

Haemodynamic and ECG variables in platelet trapping study

Baseline HR, CF, PR, and QT90 intervals, did not differ between the 4 groups (Table 2) and here (and elsewhere in the study). Coronary ligation evoked changes typical for the model, such as a fall in CF. There were no significant differences in CF or PR between groups at any time. The QT90 interval in coronary-ligated controls increased during ischemia from 53 ± 2 ms to 68 ± 3 ms then gradually fell towards the end of the experiment to 61 ± 3 ms as seen previously with this model. ¹⁹,²⁰,²⁹ QT90 changes were similar with platelet trapping, but the increase was significantly greater versus the other groups 30 min after the onset of ischemia (platelet 71 ± 4
ms; control 61 ± 3 ms; secretome 64 ± 4 ms; fluorospheres 59 ± 4 ms; p<0.05). In sham ligation controls, there were no significant effects of platelet or secretome infusion on CF, PR or QT90. The only HR variation between groups was a transient increase at the start of secretome infusion in sham (Figure 5A) and coronary ligated hearts (Figure 5B) with a weaker (non-significant) trend towards an increase in hearts with platelets trapped by ligation (Figure 5B).

**Regional ischemia activates platelets**

In Krebs solution-perfused control hearts subjected to coronary ligation, no TX was detected in coronary effluent. However in hearts with platelets trapped in the IZ, a substantial amount of TX was detected (Figure 6).

**Drug pre-treatment action on platelets, VF and haemodynamics**

Oral pretreatment with anti-platelet agents inhibited (but did not abolish) ex vivo aggregation responses to ADP (Figure 7A) and thrombin (Figure 7B). CF, HR, PR interval, QT90 interval and IZ size were similar to values in the platelet trapping study, and did not vary significantly between groups (Table 3). Ischemia-induced VF was facilitated by blood trapping compared with blood-free controls (p <0.05; Figure 8). Facilitation of VF was not reduced significantly by pretreatment in vivo with clopidogrel (P>0.05) or indomethacin (P>0.05) (Figure 8).

**Discussion**

**Overview**

Platelets trapped in the IZ by coronary ligation became activated by ischemia resulting in an increase in VF incidence in hearts in which VF risk would ordinarily be low owing to suboptimal ischemia (small IZ). Platelets were arrhythmogenic only when trapped in the IZ. Trapping of autologous whole blood mimicked the platelet effect. This was not prevented by in vivo pre-treatment with clopidogrel or indomethacin (which reduced platelet aggregability). Platelets,
platelet trapping and secretome did not lower coronary flow, precluding the possibility that platelets induced VF by evoking ischemia or rendering it more severe. This is therefore the first direct evidence that platelets facilitate VF during ischemia independently of their role in thrombosis.

**Platelets and the pathophysiological reserve for VF**

Previous reports on platelet involvement in VF are contradictory. Pretreatment with aspirin, ticlopidine, meclofenamate or indomethacin reduced ischemia-induced VF incidence from a high baseline in anaesthetized rats.\(^{15,16}\) However aspirin failed to reduce ischemia-induced VF in conscious rats with a large IZ.\(^{30}\) Likewise PAF antagonists ameliorated ischemia-induced VF in vivo in one study,\(^ {34}\) but not in others.\(^ {32,33}\) Moreover PAF and PAF antagonists possess platelet-independent effects on ischemia-induced VF.\(^ {28}\)

In most published studies the IZ was not determined. It is well established that when the IZ is large in vivo and even in Krebs solution-perfused rat hearts, all controls develop VF owing to pathophysiological reserve.\(^ {19,20,34}\) A pathophysiological reserve precludes identification of individual mediators of VF. In the present study the pathophysiological reserve was reduced by making IZ suboptimal, thereby revealing the pathophysiological ability of trapped platelets to facilitate ischemia-induced VF. The question remaining is to what extent does this knowledge inform better drug targeting of VF?

**Mechanistic insights**

The increase in QT90 interval in hearts with platelets trapped in the IZ resonates with data from De Jong et al.\(^ {18}\) who exposed human platelet secretome to rabbit cardiomyocytes and observed APD prolongation and after-depolarizations. However, other findings provide better clues to mechanisms.
Platelets were inert in non-ischemic hearts, so the mechanism of VF facilitation required an interaction with components of the ischemic milieu. Additionally, platelet sized fluorospheres were not arrhythmogenic (in shams or when trapped by ligation) confirming that VF facilitation by platelets does not operate by simple physical presence.

Trapped platelets released TX in the present study. Moreover, in 2 sham hearts (no ischemia) exogenously administered secretome caused VF. In our historical database of n>200 controls, otherwise ‘normal’ hearts do not exhibit spontaneous VF. The mechanism was primary, since secretome did not induce ischemia (no reduction in CF) in shams. The secretome effect in shams contrasts with the lack of platelet and blood effects. Conversely, secretome had little effect when trapped in the IZ, in sharp contrast to the facilitation of VF by trapped platelets and blood.

These observations provide coherent mechanistic insight: ischemia activates trapped platelets to release secretome containing a labile and short-lived arrhythmogenic mediator, sufficient to facilitate VF when IZ is suboptimal. It is important to emphasize that these mechanistic insights are tentative.

**Clinical relevance**

Owing to the complex interaction between platelets and other blood components we modified our platelet trapping approach to examine whether standard antiplatelet drugs administered *in vivo* could reduce the ability of trapped whole blood to facilitate VF *in vitro*. Clopidogrel and indomethacin were not effective in this regard. Clopidogrel reduces platelet P-selectin expression but does not prevent platelets from secreting α-granular contents; indomethacin inhibits cyclooxygenase but is not selective for TX synthesis. Moreover, despite high dosage, the effect of each drug on responses to platelet activators *in vitro*, although significant was not substantial.
Thus, standard antiplatelet drugs are unable to affect ischemia-trapped platelet function sufficiently to block facilitation of VF. This accords with their lack of benefit against SCD in humans.

**Limitations**

The isolated perfused unloaded rat heart exhibits ischemic VF that is not subject to normal mechanical or autonomic influences. Confidence that present findings have clinical relevance is limited by the extent to which the lack of loading and intact innervation may change the ability of platelets to facilitate ischemia-induced VF in suboptimal ischemia. To test this will require *in vivo* experimentation.

**Future work**

There are a large number of constituents of the platelet secretome, and identification of the one(s) necessary for facilitation of VF is beyond the scope of the present study. TXA2 is a noteworthy labile and short-lived constituent. In addition to our evidence of ischemia-specific TX release from platelets trapped in the IZ, studies with the TX synthase blockers UK38485, R-68070, and U-63557A25,37 suggest that TXA2 may facilitate ischemia-induced VF *in vivo.* Together this justifies future work to clarify links between the platelet, TX and ischemia-induced VF.

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**Conflict of Interest Disclosures:** none.
References:


<table>
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<th>Table 1. Baseline characteristics of hearts subjected to coronary ligation</th>
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<tr>
<td><strong>Base line variables</strong></td>
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<tr>
<td><strong>(value ± SEM)</strong></td>
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<tr>
<td>Heart Rate (beats/min)</td>
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<td>264.4 ± 8.9</td>
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<tr>
<td>Coronary Flow (ml/min)</td>
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<td>10.1 ± 0.7</td>
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<tr>
<td>PR interval (ms)</td>
</tr>
<tr>
<td>QT90 interval (ms)</td>
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<tr>
<td>IZ</td>
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<td>(% of ventricular mass)</td>
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Table 2. Baseline characteristics of sham hearts in the platelet trapping study

<table>
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<th>Base line variables (value ± SEM)</th>
<th>Krebs buffer N = 11</th>
<th>Platelet N = 12</th>
<th>Secretome N = 11</th>
<th>Fluorospheres N = 11</th>
<th>P-value</th>
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</thead>
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<tr>
<td>Heart Rate (beats/min)</td>
<td>285.8 ± 14.5</td>
<td>296.3 ± 12.6</td>
<td>295.5 ± 12.5</td>
<td>277.0 ± 14.7</td>
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<td>Coronary Flow (ml/min)</td>
<td>9.5 ± 0.8</td>
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<td>9.6 ± 0.5</td>
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<td>PR interval (ms)</td>
<td>40.4 ± 1.4</td>
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<td>QT90 interval (ms)</td>
<td>56.5 ± 3.2</td>
<td>56.5 ± 1.7</td>
<td>55.1 ± 1.9</td>
<td>52.1 ± 2.9</td>
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Table 3. Baseline characteristics of hearts subjected to coronary ligation in the blood trapping study

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<th>Blood N = 8</th>
<th>Clopidogrel N = 9</th>
<th>Indomethacin N = 14</th>
<th>P-value</th>
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<td>Heart Rate (beats/min)</td>
<td>304.3 ± 7.3</td>
<td>290.9 ± 9.1</td>
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<td>Coronary Flow (ml/min)</td>
<td>9.9 ± 0.4</td>
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<td>11.4 ± 0.6</td>
<td>10.2 ± 0.8</td>
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<td>PR interval (ms)</td>
<td>36.6 ± 1.3</td>
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<td>39.9 ± 0.9</td>
<td>40.8 ± 1.8</td>
<td>0.74</td>
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<td>QT90 interval (ms)</td>
<td>54.8 ± 1.5</td>
<td>63.4 ± 2.5</td>
<td>57.8 ± 3.1</td>
<td>60.7 ± 4.8</td>
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<tr>
<td>IZ (% of ventricular mass)</td>
<td>31.6 ± 2.0</td>
<td>35.0 ± 1.3</td>
<td>31.0 ± 1.5</td>
<td>32.4 ± 0.1</td>
<td>0.38</td>
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</table>

Figure Legends:

Figure 1. Novel cannula engineered for direct platelet delivery to the isolated heart. A modified Langendorff cannula was made from stainless steel and modified to receive a sidearm to allow
passage of an inert 1 mm internal diameter polypropylene tube to the tip of the cannula. The shear rate within the sidearm tubing was sub-arteriolar (<1000 s⁻¹)

**Figure 2.** Platelet aggregation studies confirm platelet viability and functionality. Washed rat platelets were derived by standard centrifugation, and re-suspended at a concentration of 2 x 10⁸ /ml. Aggregation traces demonstrated normal dose response curves to agonist stimulation with collagen, ADP and thrombin in Tyrode solution (A-C) or Krebs solution (D,E). In part E, X refers to the time point at which 500 µl of washed platelets were perfused through the modification to the cannula at 100 µl/s. All traces are representative of n = 3.

**Figure 3.** The delivery of platelets is sufficient to cause platelet trapping. To validate the platelet delivery/trapping method, rat hearts were perfused with platelets radiolabelled with 50MBq ¹¹¹In oxine sulphate. After coronary ligation hearts were frozen in liquid nitrogen for autoradiography performed on 20 micron sections. Platelets were trapped by coronary ligation in the IZ (in the left ventricle-LV) and excluded (very little adhesion) in the remote region (right ventricle-RV). Representative image of n = 3.

**Figure 4.** Platelet trapping facilitates ischemia-induced VF. Incidence of VF evoked by 30 min regional ischemia in the Langendorff perfused rat heart. Platelets, secretome or fluorospheres were delivered for 20 min beginning 10 min before the start of ischemia. *p<0.05 versus controls.

**Figure 5.** Secretome infusion resulted in fluctuations in HR. Rat platelet secretome (0.21mg/mL protein) in a final volume of 4 ml of warmed gased Krebs solution was delivered at 0.2 ml/min
for 20 min, with coronary ligation (or sham ligation) performed at the half way point (10 min).

Secretome consistently increased HR (defined as any change sustained for at least 10 sec) compared with the maximum changes during the equivalent time interval in other groups of sham (part A) or coronary ligated (part B) hearts. * p<0.05 versus controls.

**Figure 6.** Regional ischemia activates platelets. Rat hearts underwent 10 min ischemia or sham ligation. In two groups platelets were delivered for 20 min beginning 10 min before the start of ischemia or sham ligation. TXB2 was measured by ELISA in the coronary effluent collected during the first minute after cessation of infusion of platelets (ligation or sham)* p<0.05 versus ‘Krebs ligation’ (no platelets).

**Figure 7.** Effects of drug pre-treatment on platelet aggregation. Rats were pre-treated with indomethacin or clopidogrel for a minimum of 5 days. Washed platelets were derived by standard centrifugation and re-suspended in Krebs solution at 2 x 10⁸/ml. Platelet aggregation responses to (A) ADP (n = 3) and (B) thrombin (n = 3) are shown. *p<0.05 versus controls.

**Figure 8.** Incidence of VF in anti-platelet drug pre-treated study. Incidence of VF evoked by 30 min regional ischemia in the Langendorff perfused rat heart. Rats were pretreated for at least 5 days *in vivo* with drug or vehicle. Autologous blood was delivered for 20 min beginning 10 min before the start of ischemia. Blood trapping was associated with a high incidence of VF (*P<0.05 versus control hearts without blood trapping). VF incidence with blood trapping was not significantly reduced by clopidogrel or indomethacin pretreatment *in vivo.*
(X) 500 μl of washed platelets perfused through modification at 100 μl/s.
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