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Disparate effects of adhesion and degranulation of platelets on myocardial and coronary function in postischaemic hearts

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Abstract

Objective: Beside the major effect of acute thrombus formation, little is known about the interaction of platelets with the coronary endothelium in an ischaemia–reperfusion situation. The present study was designed to investigate, separately, the consequences of platelet adhesion and degranulation during myocardial reperfusion. **Methods:** Isolated guinea pig hearts perfused with Krebs–Henseleit buffer and performing pressure–volume work were used. We infringed myocardial function by imposing ischaemia (20 min of low-flow perfusion with 1 ml/min and 10 min of global ischaemia) and reperfusion (15 min with 5 ml/min). During low-flow perfusion, the coronary endothelium was stimulated by thrombin before and during infusion of a bolus: 10^8 washed human platelets ± the Arg–Gly–Asp (RGD) analogon lamifiban, the supernatant of 10^8 thrombin-stimulated platelets, fibrinogen (2 μM), lamifiban (2 μM) or Tyrode's solution (control group). The parameter external heart work (EHW), determined pre- and postischaemically, served as criterion for recovery of myocardial function. Additionally, the formation of capillary transudate was measured during the reperfusion phase to assess coronary permeability. Coronary perfusion pressure was monitored continuously and myocardial production of lactate and consumption of pyruvate were measured. Electron microscopy of hearts was performed after platelet application to verify platelet adhesion in the coronary system. **Results**: Recovery of EHW by hearts without platelet application was 64 ± 3% and was significantly reduced to 49 ± 5% by platelet infusion ($n = 8$ each). Infusion of supernatant of thrombin-stimulated platelets did not impair recovery of heart work. In the reperfusion phase (6th–10th min), hearts that either had received platelets or supernatant of platelets exhibited a significantly reduced production of capillary transudate (70 μl/min vs. 180 μl/min for the controls). Intracoronary bolus application of fibrinogen or lamifiban also reduced coronary leak. Coronary perfusion pressure and metabolic parameters were not statistically different between the groups at any time. **Conclusions**: Platelet adhesion to the coronary endothelium in a situation of myocardial ischaemia impairs cardiac recovery, whereas constituents released by platelets may have beneficial effects on the integrity of the coronary endothelium. In particular, fibrinogen seems to contribute to the permeability reducing effect, possibly by interaction with endothelial receptors recognising the RGD sequence.

Keywords: Adhesion; Capillary leakage; Fibrinogen; Ischemia; Lamifiban; Platelets; Reperfusion; Guinea pig hearts

1. Introduction

Myocardial damage due to coronary occlusion and consecutive reperfusion is a rather common clinical occurrence. Reperfusion injury may result from unstable angina, acute thrombolysis after myocardial infarction, percutaneous coronary angioplasty, bypass surgery and heart transplant reperfusion. The relevance of adhesion of polymorphonuclear neutrophils (PMN) to the activated coronary endothelium was shown for the development of acute postischaemic damage [1–3]. Beside the possibility of microcirculatory disturbances, production of oxygen radicals by adherent PMN further deteriorates endothelial integrity and myocardial performance [4].

With respect to platelets, acute thrombus formation is a well-known cause of serious reperfusion complication, adhesion of platelets to the coronary endothelium being the

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initial step. Platelets are a rich source of a wide range of biologically active materials that are capable of inducing and augmenting certain inflammatory responses. Such materials have been shown to be both, preformed mediators stored in the granula (e.g. ADP, fibrinogen, serotonin, histamine, PF_4) [5] and newly synthesised agents resulting from the perturbation of membrane phospholipids (e.g. TXA_2, $PGF_{2\alpha}$, PGE_2). Furthermore, activation of an IgE receptor of platelets has been shown to result in the production of cytotoxic free radicals [6]. Not surprisingly, there have been reports that intracoronary infusion of platelets in an ischaemia–reperfusion situation can induce arrhythmias [7] and impair recovery of heart performance [8,9]. However, a number of studies were able to demonstrate cardioprotective effects of platelet infusion during myocardial reperfusion [10–12]. These conflicting results may be due to differences in the experimental protocol (e.g. absence or presence of exogenous stimuli) and opposing effects of platelet adhesion and platelet secretion. The present study was designed to investigate separately the consequences of adhesion and degranulation of platelets during myocardial ischaemia and reperfusion. The experiments were performed on isolated perfused hearts of guinea pigs using washed human platelets in defined number (10^8), infused into the coronary system under strictly standardised conditions.

2. Methods

2.1. Reagents

Iloprost was produced by Schering, Berlin, Germany. Human thrombin and fibrinogen type I were supplied by Sigma, St. Louis, USA. Lamifiban, a thyrosine derivate, was kindly provided by Hoffmann-La Roche, Basel, Switzerland. Lamifiban is a highly selective non-peptide GPIIb/IIIa antagonist, which binds to the Arg–Gly–Asp (RGD) recognition sequence of the GPIIb/IIIa receptor. Thrombin, fibrinogen and lamifiban were dissolved in H_2O for infusion. All other reagents were purchased from Merck, Darmstadt, Germany.

2.2. Solutions

Phosphate-buffered saline (PBS), pH 7.4, contained (in mmol/l): NaCl, 120; KCl, 2.7; Na_2HPO_4, 4.0; KH_2PO_4, 5.0 and 0.1% ethylenediaminetetraacetate (EDTA).

Tris-buffered Tyrode's solution, pH 7.4, contained (in mmol/l): NaCl, 137; KCl, 2.6; $MgCl_2 \times 6H_2O$, 1.0; $CaCl_2 \times 2H_2O$, 3.0; Tris, 1.0 and 0.1% glucose.

The composition of the Krebs–Henseleit buffer was as follows (in mmol/l): NaCl, 126; $NaHCO_3$, 24; KCl, 4.7; $MgSO_4 \times 7H_2O$, 0.6; $CaCl_2 \times 2H_2O$, 1.25; KH_2PO_4, 1.2; pyruvate, 0.3; glucose, 5.5 and insulin, 5 IU/l.

Cellwash (Becton Dickinson, San Jose, USA) is a phosphate-buffered sodium chloride solution for preparation and analysis of cells in the flow cytometer.

2.3. Platelet preparation

Blood from 9 healthy adult volunteers who had not ingested aspirin or other anti-inflammatory drugs within the previous 10 days was drawn by venipuncture into polypropylene syringes containing EDTA 0.1% and Iloprost 50 ng/ml (final concentrations) for anticoagulation. The blood was centrifuged at $200 \times g$ for 15 min. The platelet rich plasma was separated and centrifuged at $2000 \times g$ for 10 min. After discarding the supernatant, the pellet of platelets was twice resuspended in PBS and centrifuged at $10\,000 \times g$ for 1 min. The washing procedure served to remove Iloprost and plasma components such as fibrinogen, which mediate platelet aggregation and would, thereby, confound the interpretation of intracoronary sequestration data. Finally, washed platelets were resuspended in Tyrode's solution and adjusted to a final concentration of $100\,000/\mu l$. The adjusted platelet suspensions were drawn into a polypropylene syringe immediately before intracoronary application. To determine platelet numbers in Tyrode's solution and in samples of venous effluent, specimens were counted in triplicate with a Coulter counter ZM and analysed for distribution of platelet diameters in a Coulter Channelyzer 256 (Coulter Electronics, Luton, UK).

2.4. Heart preparation

The care of the animals and all experimental procedures were in full accordance with German animal protection laws and officially approved.

Hearts were isolated from male guinea pigs (body weight 200–300 g), following cervical dislocation, without use of any anticoagulants or anaesthetics. After median thoracotomy and rapid arrest of the beating hearts by superfusion with ice-cold isotonic saline, the ascending aorta was cannulated and the hearts were rapidly excised. The isolated organs were perfused in the non-working 'Langendorff' mode at 37°C with a modified Krebs–Henseleit buffer, equilibrated with 94.4% O_2 and 5.6% CO_2 (pH 7.40 ± 0.05) for 15–20 min to allow further preparation. The veins entering the right atrium and the pulmonary veins were ligated to ensure that the coronary effluent passed through the pulmonary artery. This vessel was cannulated to enable collection of the coronary venous effluent. The left atrium was cannulated through an opening formed by uniting the four pulmonary vein orifices to allow natural filling of the atrium and contraction of the left atrial appendage [13]. The perfusion pressure was continuously monitored with a pressure transducer (FMI GmbH, Egelsbach, Germany) and aortic and coronary flows were recorded with an ultrasonic flow meter (Trans-

sonic Systems, Ithaca, USA). Heart rate was derived from the phasic perfusion pressure signal.

The hearts performed pressure–volume work at a left atrial filling pressure of 8.8 mmHg and a mean aortic pressure of 59 mmHg. Pre- and afterload were regulated by resistance valves in the filling system of the left atrium and the outflow system of the aorta, respectively.

2.5. Experimental protocol for working hearts

The perfusion protocol is outlined in Fig. 1. After an initial 20 min work phase, W_1, the hearts were perfused in a non-working, constant flow mode. After closure of both the in- and outflow valves of the hydrostatically controlled perfusion system, a pump supplied a constant flow 'retrogradely' into the coronary system via the aortic cannula. A reduced coronary flow of 1 ml/min was established for 20 min to mimic 'low-flow' perfusion. Thereafter, a global stopped-flow ischaemia was imposed for 10 min. The temperature of the hearts in these two phases was kept constant at 37°C by immersion in warm Tyrode's solution with a pH of 7.4. Myocardial reperfusion was established at constant coronary flow of 5 ml/min for the next 15 min, whereafter work phase W_2 was performed (20 min) under conditions identical to those of W_1.

The following experimental groups were investigated: (1) ischaemia-control (no application of platelets, $n = 8$); (2) platelets (application of platelets during low-flow perfusion, $n = 8$); (3) supernatant (application of supernatant of thrombin-stimulated platelets during low-flow perfusion, $n = 5$); (4) lamifiban (application of platelets during low-flow perfusion in the presence of 1 μM lamifiban, $n = 5$); and (5) time-control hearts, perfused with 5 ml/min coronary flow for 45 min without inducing ischaemia ($n = 5$); W_1 and W_2 being performed as in the other cases.

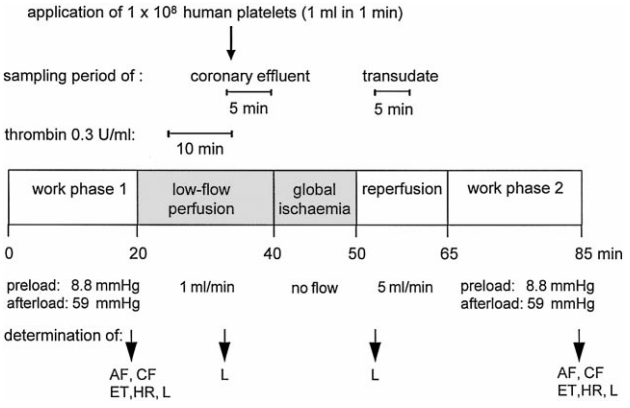

Fig. 1. Schematic presentation of the standard experimental protocol. Hearts performed volume–pressure work for 20 min each at the beginning and at the end of the protocol. After work phase W_1, the coronary system was perfused at constant volume in a low-flow phase (1 ml/min), followed by a global ischaemia (no flow) and a reperfusion phase (5 ml/min). In the 15th min of the low-flow phase, a platelet bolus was applied (1 ml in 60 s, 100000/μl perfusate). Coronary effluent and epicardial transudate were sampled during the periods indicated in the figure. During low-flow perfusion, endothelium was temporarily stimulated with thrombin. AF, aortic flow; CF, coronary flow; ET, ejection time; HR, heart rate; L, effluent sample for lactate and pyruvate analysis.

With the exception of the time-control group, all hearts were stimulated with thrombin (0.3 U/ml) from the 6th to the 15th min of the low-flow phase.

In the 15th min of low-flow perfusion a 1 ml bolus of platelets suspended in Tyrode's solution (100000/μl) was applied over 1 min into the coronary system via the aortic cannula with an infusion pump. To keep coronary flow constant during infusion of platelets, perfusion with Krebs–Henseleit buffer was stopped for that time. Previous experience had shown that platelet adhesion in our experimental setting is facilitated by reduced flow rate.

To differentiate the effect of platelet adhesion from that of platelet degranulation on the performance of heart work in our ischaemia–reperfusion situation, a group was established using supernatant of activated platelets. One ml of platelet suspension (100000/μl in Tyrode's solution) was stimulated with 0.3 U/ml human thrombin for 10 min. Platelets were then pelleted for 10 min at $13000 \times g$ and the resultant supernatant was infused during low-flow perfusion in the identical manner as platelet suspensions (1 ml in 60 s).

Haemodynamic variables that were determined at the end of W_1 and W_2 were coronary and aortic flow, spontaneous heart rate and ejection time of stroke volume. External heart work (the sum of pressure–volume work and acceleration work) was calculated from these variables [13]. Because preload and afterload were held constant for all preparations during phases of work, changes in cardiac performance were reflected largely as alterations in cardiac output. Recovery of external heart work was defined as postischaemic compared to preischaemic external heart work, each at 20 min, and expressed in percent.

In the reperfusion period epicardial fluid (transudate) was collected from the apex of the hearts over 5 min (6th–10th min of reperfusion). Transudate formation reflects net fluid passage across the vessel walls in the coronary bed.

As a measure of the severity of myocardial ischaemia, cardiac lactate release and pyruvate consumption were measured each time in the 20th min of W_1 and W_2, the 14th min of the low-flow perfusion phase and the 5th min of reperfusion. Lactate and pyruvate in the coronary effluent (aliquots of about 1 ml) were determined using HPLC [14]. Pyruvate consumption and lactate release were calculated as follows:

$$\text{Pyruvate consumption} = (\text{pyruvate}_{\text{in perfusate}} - \text{pyruvate}_{\text{in effluent}}) \times \text{perfusion rate};$$

$$\text{Lactate release} = \text{lactate}_{\text{in the effluent}} \times \text{perfusion rate}.$$

The ratio of lactate production to pyruvate consumption was then calculated, because this quotient emphasises anaerobic metabolism.

2.6. Experimental protocol for exclusive measurements of transudate formation

To study the formation of transudate in the reperfusion phase in the course of time, the volume-constant reperfusion phase was extended from 15 to 30 min, i.e. no second work phase was performed. From the 6th to the 30th min of reperfusion, the formation of transudate was measured in intervals of 5 min. Values were expressed as μl/min for the corresponding interval. Four groups of hearts ($n = 6$ per group) were investigated: (1) ischaemia-control (no platelets were applied); (2) platelets (10^8 washed platelets were infused in the low-flow phase as described above); (3) fibrinogen (to check the influence of fibrinogen secreted by platelets on transudate formation, human fibrinogen type I in a concentration of 2 μM was infused instead of the platelet suspension, but in the same manner (1 ml in 1 min in the 15th min of low-flow perfusion)); and (4) Lamifiban (lamifiban, a non-peptide agent with highly selective binding to RGD-recognising receptor sequences, tested by applying a concentration equimolar to that of fibrinogen). All hearts received thrombin (0.3 U/ml) in the above-described manner.

2.7. Adhesion measurements

During low-flow perfusion a 1-ml bolus of platelets suspended in Tyrode's solution (100 000/μl) was applied into the coronary system over 1 min. To quantify the number of platelets leaving the coronaries (platelet output), coronary effluent was collected continuously during the 60 s of bolus application and in the following 240 s. Pilot studies had shown that only a negligible number of the applied platelets (< 1%) emerged after such a 300-s sampling period. Coronary effluent was sampled in ice-cold 10% formaldehyde solution (end concentration 1–2%) to fix cells for counting and flow cytometric analysis. Immediately before each intracoronary application, a test bolus of platelets of equal volume and duration (1 ml in 60 s) was sampled to determine the number of cells actually leaving the syringe (platelet input). The percentage of platelets adherent to the endothelium was calculated as follows:

$$\text{Adhesion}\,(\%) = (1 - (\text{platelet output}/\text{platelet input})) \times 100$$

To test the involvement of the platelet adhesion molecule GPIIb/IIIa in the interaction with the coronary endothelium, lamifiban in a concentration of 1 μM was added to the platelet suspension 10 min prior to intracoronary infusion. The IC_{50} for lamifiban in ADP-induced (10 μM) platelet aggregation is 87 nM [15]. In in vitro experiments, lamifiban showed very potent anti-aggregatory effects at the concentration of 1 μM [16].

2.8. Monoclonal antibodies

Mouse anti-human CD41a (Camon, Wiesbaden, Germany), labelled with FITC, detects the GPIIb/IIIa complex in its resting and activated forms.

Mouse anti-human CD62P (Harlan Sera-Lab, Crawley Down, UK), labelled with R-PE, recognises the α-granule membrane glycoprotein P-selectin.

Mouse anti-human CD42b (Southern Biotechnology Associates, Birmingham, USA), labelled with FITC, binds to the GPIb molecule, which is a von Willebrand's factor-dependent adhesion receptor.

2.9. Flow cytometry

Formalin-fixed platelets of the test bolus and of the coronary effluent were analysed in a *FACScan* flow cytometer (Becton Dickinson, San Jose, USA) for glyco-

Table 1
Initial (W_1) and postischaemic (W_2) haemodynamic values of hearts successively subjected to 20 min of low-flow ischaemia, 10 min of global ischaemia and 15 min of reflow

Group		Coronary flow (ml/min)	Aortic flow (ml/min)	Heart rate (min^{-1})	EHW (mJ/min)
Time-control	W_1	9.0 ± 0.3	49.1 ± 2.7	245 ± 6	400 ± 20
Time-control	W_2	8.0 ± 0.2	46.5 ± 3.6	232 ± 3	370 ± 25[b]
Ischaemia-control	W_1	9.4 ± 0.4	50.2 ± 1.6	245 ± 5	410 ± 10
Ischaemia-control	W_2	6.2 ± 0.4	32.3 ± 2.0	232 ± 6	260 ± 20[a,b]
Platelets	W_1	10.5 ± 0.3	49.9 ± 3.0	244 ± 3	410 ± 20
Platelets	W_2	7.8 ± 0.5	23.7 ± 4.3[a]	226 ± 6	210 ± 30[a,b]
Supernatant	W_1	9.6 ± 0.5	48.7 ± 2.8	232 ± 6	400 ± 20
Supernatant	W_2	7.8 ± 0.3	34.7 ± 3.6	223 ± 5	290 ± 30[a,b]
Lamifiban + platelets	W_1	9.1 ± 0.1	49.6 ± 3.6	245 ± 7	400 ± 30
Lamifiban + platelets	W_2	6.7 ± 0.2	31.9 ± 2.6	228 ± 7	260 ± 20[a,b]

The following boli were applied in the 15th min of low-flow perfusion: ischaemia-control, Tyrode's solution; platelets, 10^8 washed platelets; supernatant, the supernatant of 10^8 thrombin-stimulated platelets; lamifiban, 10^8 washed platelets plus 1 μM lamifiban. The time-control group was not exposed to ischaemia, but perfused for the same time period with a coronary flow of 5 ml/min. Values are means ± s.e.m. ($n = 5$–8). EHW, external heart work.
[a] $P < 0.05$ vs. time-control group.
[b] $P < 0.05$ vs. the W_1 value of the corresponding group (paired *t*-test).

protein expression by using labelled monoclonal antibodies: anti-GPIIb/IIIa, anti-P-selectin and anti-GPIb. In general, samples containing about 5×10^6 platelets were centrifuged at $1200 \times g$ for 15 min and the supernatant was discarded. Platelets were resuspended in 100 μl Cellwash and incubated 15 min after the addition of 5 μl of monoclonal antibody. Two ml of Cellwash were then added for dilution of the antibodies and platelets were centrifuged again at $1200 \times g$ for 15 min. The pellet was resuspended in 500 μl Cellwash and immediately analysed in the flow cytometer. Antibody binding was expressed as relative mean particle fluorescence intensity of total platelet population and was used as a quantitative measure of glycoprotein surface expression before and after coronary passage. For each measurement, negative controls were performed, in which binding of non-specific monoclonal antibodies labelled with FITC or PE to platelets fixed after coronary passage was assayed.

2.10. Electron microscopy

To evidence adherence of platelets and to characterise the location of platelets in the coronary system after platelet application in the low-flow phase, electron microscopy of guinea pig hearts was performed. At the end of the low-flow phase these hearts were perfused with 3% glutaraldehyde (pH 7.4) at 4 ml/min for 15 min. Afterwards, both atria were removed and the ventricles were cut into four pieces. These were fixed in 3% glutaraldehyde for 2 h and then stored in 1% glutaraldehyde until processed further. A 2-h postfixation episode in 2% OsO_4 was followed by dehydration in a series of graded ethanols and embedding in Araldite. For orientation, 1-μm-thick sections were stained with Toluidine blue. Ultrathin sections were cut on a Reichert ultramicrotome, contrasted for 5 min with uranylacetate and lead citrate, and viewed in a Philips CM 10 electron microscope.

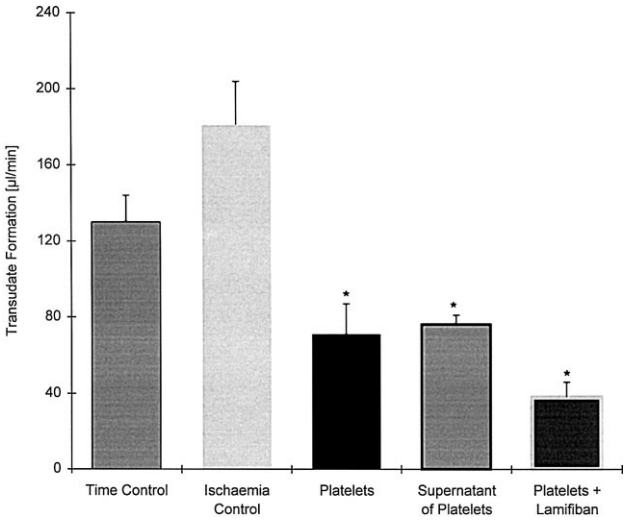

Fig. 3. Transudate (epicardial fluid) was collected from the apex of the heart over a 5-min period in the reperfusion phase (6th–10th min). The time-controls were perfused at constant volume without inducing ischaemia. The ischaemia-control group was subjected to low-flow and global ischaemia. After application of either 10^8 washed platelets, the supernatant of 10^8 thrombin-stimulated platelets or 1 μM lamifiban plus 10^8 platelets in the low-flow phase the formation of transudate was significantly decreased in comparison to the ischaemia-control ($^*P < 0.05$). Values are means ± s.e.m., $n = 5–8$.

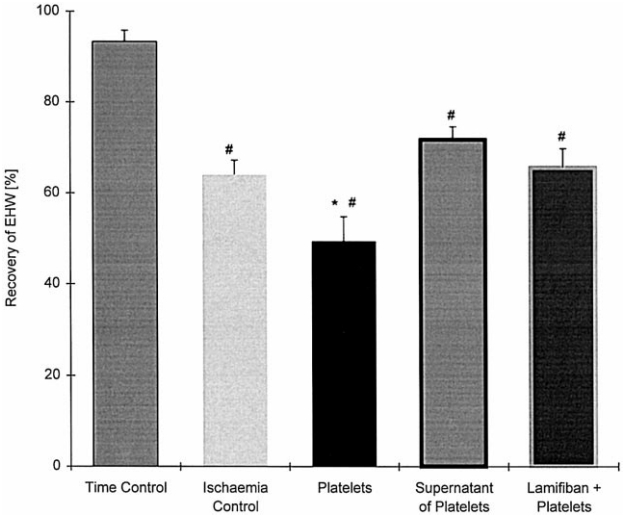

Fig. 2. Recovery of external heart work (EHW). The left column shows the time-control hearts which were not subjected to ischaemia. The ischaemia-control group was subjected successively to low-flow and global ischaemia. After application of 10^8 washed platelets, recovery of EHW was significantly impaired, but not after infusion of supernatant of thrombin-stimulated platelets or of washed platelets in the presence of 1 μM lamifiban. $^*P < 0.05$ vs. all other groups; $^\#P < 0.05$ vs. time-control group. Values are means ± s.e.m., $n = 5–8$.

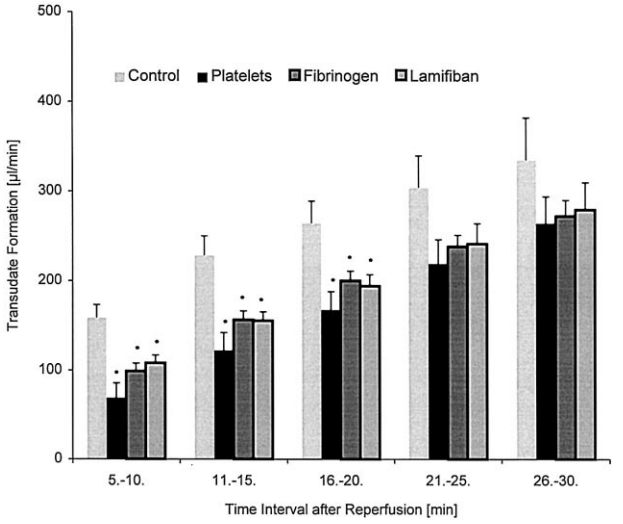

Fig. 4. Time course of transudate formation in the extended reperfusion phase (30 min). Transudate was sampled in 5-min periods. Application of 10^8 washed platelets, fibrinogen (2 μM), or lamifiban (2 μM) in the low-flow phase reduced the production of transudate after reflow in a statistically significant and long-lasting manner. Values are means ± s.e.m., $n = 6$ per group. $^*P < 0.05$ vs. the control group.

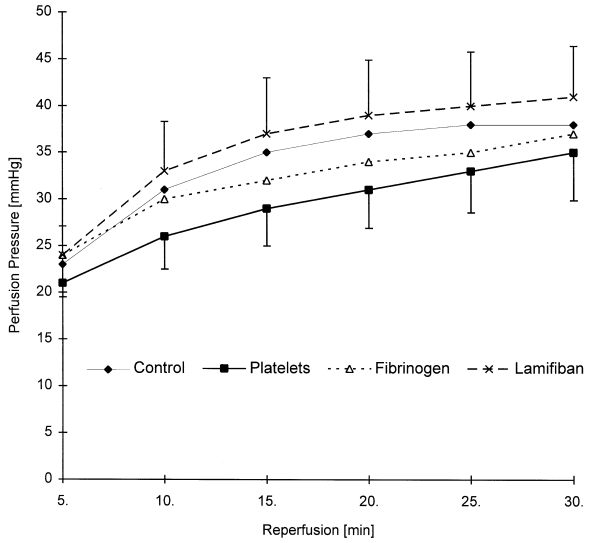

Fig. 5. Time course of coronary perfusion pressure (mmHg) in the extended reperfusion phase (30 min). A slight increase over the course of time is visible in all groups, but no statistically significant differences were detectable between groups. Values are means ± s.e.m., $n = 6$ per group.

2.11. Statistical methods

The results are expressed as means ± s.e.m. For comparisons between two groups, Student's *t*-test was used. For comparison of several groups, analysis of variance (ANOVA) was first performed to detect a possible overall difference. Whenever a significant effect was obtained, we performed multiple comparison tests between the groups using Student–Newman–Keul's test (Sigma Stat 1.0, Jandel). Differences between data were considered significant at $P < 0.05$.

3. Results

3.1. Recovery of external heart work and adhesion of platelets

Intracoronary adhesion of platelets was 16.7 ± 2.4% under low-flow conditions in the platelet group. In the presence of 1 μM lamifiban, the adhesion of platelets was reduced to 10.6 ± 2.3%, although not to a statistically significant extent. Adhesion of platelets in hearts not exposed to low-flow ischaemia amounted to about 3% (data not shown).

The pre- and postischaemically measured haemodynamic data of the working hearts are shown in Table 1. The performance of time-control hearts proved to be stable. EHW was reduced about 25% by successively imposing low-flow and global ischaemia (Fig. 2). Application of platelets (10^8) during the low-flow phase reduced EHW to a statistically significant extent, recovery being about half that of time-controls. On the other hand, application of the supernatant of 10^8 platelets prestimulated with thrombin gave a recovery of EHW similar to the ischaemia-control group (Fig. 2). In the presence of lamifiban, the reduced percentage of adherent platelets (see above) was no longer able to measurably impair cardiac function, giving a recovery of EHW as good as the ischaemia-control group.

3.2. Transudate formation

Formation of transudate on the epicardial surface of the isolated heart is related to endothelial integrity and coronary perfusion pressure. Transudate formation measured in time-control hearts perfused at constant volume (5 ml/min) was 130 ± 14 μl/min over a 5-min sampling period. This value was elevated in hearts subjected to the ischaemia–reperfusion protocol, amounting to 180 ± 23 μl/min from the 6th to the 10th min of reperfusion. Infusion of either platelets, platelets plus lamifiban, or the supernatant of stimulated platelets in the low-flow phase led to a highly significant reduction of transudate formation as measured between the 6th and the 10th min of reperfusion versus the ischaemia-control group (Fig. 3).

With regard to the time course of transudate production in the extended reperfusion phase, the data in Fig. 4 demonstrate an immediate and long-lasting inhibitory effect of platelet application on capillary leakage. Until the 20th min of the reperfusion phase, transudate formation in the platelet, the fibrinogen and the lamifiban group remained significantly reduced in comparison to the ischaemia-control group, with no differences between the three interventions. After the 20th min of reperfusion, there was still a tendency towards reduced transudate formation in the three treatment groups, but statistical significance was lost. This suggests a gradual loss of the

Table 2
Ratio of lactate production to pyruvate consumption

Group	W_1 (20th min)	Low-flow (14th min)	Reperfusion (5th min)	W_2 (20th min)
Ischaemia-control	0.7 ± 0.2	14 ± 1.5[a]	4.0 ± 0.5[a]	2.1 ± 0.1[a]
Platelets	0.7 ± 0.1	13 ± 0.7[a]	5.1 ± 0.9[a]	1.7 ± 0.4[a]
Supernatant	0.8 ± 0.1	12 ± 0.8[a]	4.2 ± 0.5[a]	1.8 ± 0.2[a]

Coronary effluent was analysed for lactate and pyruvate by HPLC. Values are means ± s.e.m. ($n = 5–8$). No statistically significant differences between the groups were detected in any perfusion phase.
[a] $P < 0.05$ vs. the value in W_1 of the corresponding group.

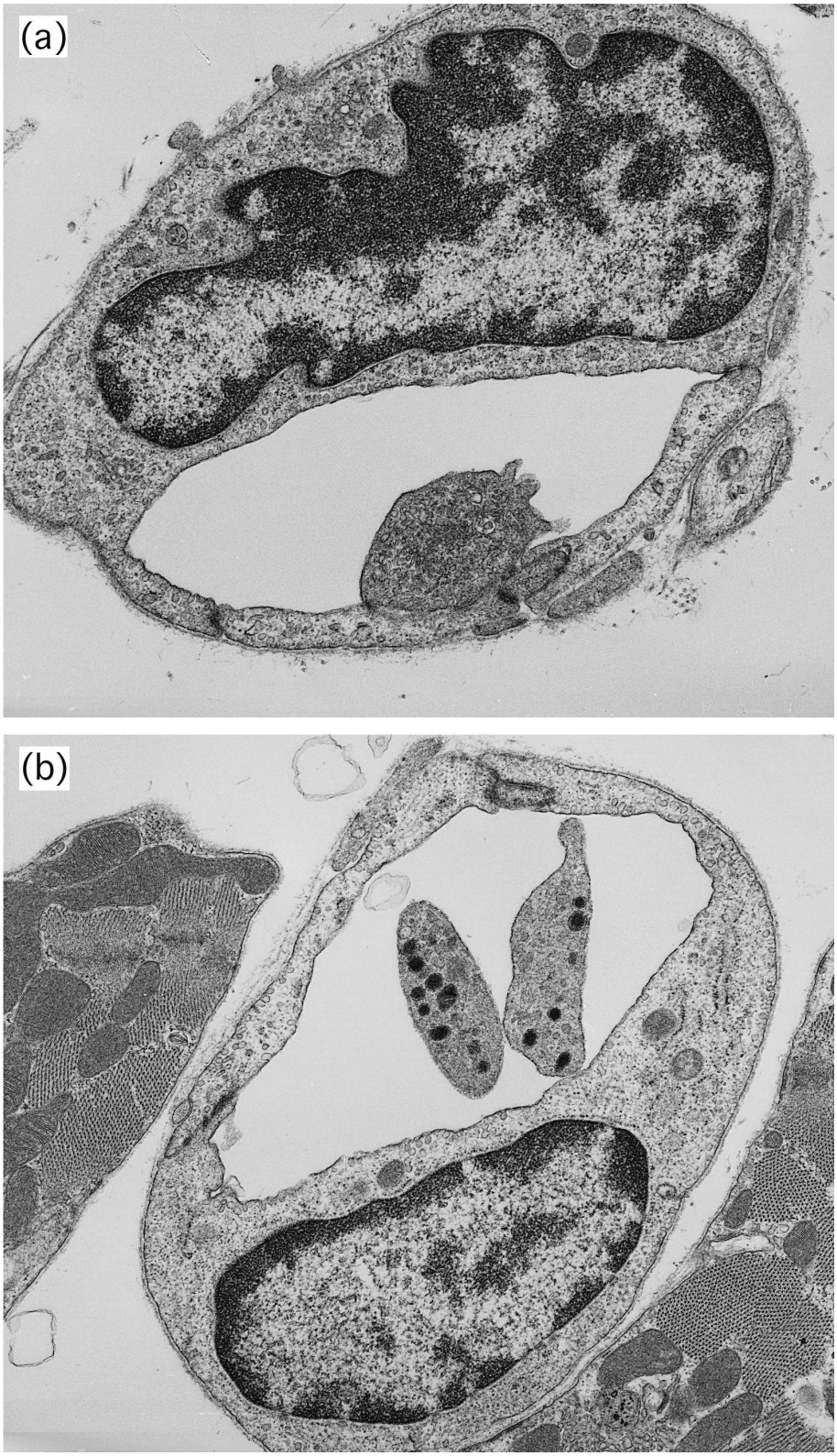

Fig. 6. Two sections of a guinea pig heart fixed after application of human platelets, as viewed by electron microscopy (1:17 700). One (A) and two (B) adherent platelets are located in a coronary capillary vessel surrounded by myocardium (B). The adherent human platelets still contain their α- and dense granula. No aggregate formation by platelets was visible.

sealing actions on capillary leakage. Interestingly, transudate formation increased in parallel to the perfusion pressure (see Fig. 5) over the extended reperfusion period, in all groups.

3.3. Coronary perfusion pressure

Coronary perfusion pressure (CPP) after 12 min of low-flow perfusion (1 ml/min) was in the order of 4–6 mmHg and not significantly different among the groups. CPP did not change after application of the platelet bolus. In the subsequent reperfusion phase, CPP rose continuously from values (means) of 20–25 mmHg in the 5th min to 35–40 mmHg in the 30th min (Fig. 5). However, at no time were there statistically significant differences between the groups.

3.4. Production of lactate and consumption of pyruvate

Lactate release in W_1 averaged about 1 μmol/min and rose significantly in each group to around 2 μmol/min in W_2. During low-flow perfusion release was in the range (means) of 2.6–3.0 μmol/min and decreased to 1.3–2.3 μmol/min 5 min after the start of reperfusion. There were no statistically significant differences between groups. Consumption of pyruvate in W_1 averaged 1.5 μmol/min and decreased slightly, but significantly, in each group to around 1 μmol/min in W_2. During low-flow perfusion, pyruvate consumption decreased to 0.2 μmol/min and increased again to about 0.4 μmol/min in the 5th min of reperfusion (no significant differences between the groups). The ratio of lactate production to pyruvate consumption revealed a strongly anaerobic metabolic situation of the hearts during low-flow perfusion (ratio 12–14) and reperfusion (ratio 4–5) in comparison to W_1 and W_2 (ratio 0.7–2.1), but, again, the groups showed no statistically significant differences (see Table 2).

3.5. Platelet surface glycoproteins

Measurements were performed of surface glycoproteins (GPIIb/IIIa, P-selectin and GPIb) of platelets before and after passage of the coronary system. Means ± s.e.m. of the relative immunofluorescence intensity before/after coronary passage were as follows: GPIIb/IIIa, 140 ± 12/122 ± 14; P-selectin, 48 ± 4/42 ± 3; GPIb, 30 ± 2/27 ± 2. Thus, a decrease of about 10% of the relative immunofluorescence intensity during coronary passage was detected for all three glycoproteins. For GPIb and GPIIb/IIIa, but not for P-selectin, the decrease attained statistical significance (paired *t*-test).

3.6. Electron microscopy

Of the inspected coronary vessels, 90–95% were totally free of platelets. In the remaining 5–10% of the vessels, mainly single and, very occasionally, groups of two or three adherent platelets were found (Fig. 6). Larger aggregates were not encountered. Adherent platelets did not occlude the vessel lumen, in any case. Interestingly, adherent platelets were located in capillaries and small venules, whereas arterioles were free of platelets. Granula contents were still visible in most of the adherent platelets.

4. Discussion

In this study of isolated guinea pig hearts, sequential low-flow ischaemia and global ischaemia followed by reperfusion resulted in a marked decrease in performance of external heart work. The cardiac dysfunction was significantly more pronounced after application of just 10^8 washed human platelets in the low-flow phase, about 17% of which were sequestered in the coronary microvasculature. There was no evidence of actual coronary occlusion by platelets. These results demonstrate that platelet adhesion to the coronary endothelium in a situation of myocardial ischaemia can impair recovery of heart performance. In contrast, degranulation of platelets may have beneficial effects on the integrity of the coronary endothelium in the reperfusion situation. This was evidenced by a quantitatively identical reduction in net coronary leak of hearts treated either with platelets or with the supernatant of an equal number of thrombin-stimulated platelets. Release of fibrinogen from platelets seems to contribute in part to this cardioprotective effect, because it was mimicked both by fibrinogen and by lamifiban, a non-peptide RGD sequence analogon.

The use of human platelets instead of guinea pig platelets in our xenogenic model offers several advantages: (1) monoclonal antibodies against surface glycoproteins are available for human platelets, not for those of guinea pigs; (2) using human blood, we were able to reduce the number of animals required for the experiments substantially, as no blood donor animals were needed; (3) the influence of drugs, e.g., lamifiban, on human platelets puts our experimental setting closer to the clinical situation, our ultimate point of interest. Although caution may be warranted with respect to the absolute extent of platelet adhesion seen, in previous studies with granulocytes, we have already demonstrated that a qualitatively identical sequestration occurs after infusion of guinea pig or human granulocytes in the isolated guinea pig heart [17,18].

4.1. Platelet adhesion

About 17% (corresponding to 1.7×10^7) of the platelets applied into the coronary system during low-flow perfusion did not re-emerge from the hearts.

There are several lines of evidence that adhesion, but not aggregation, of platelets in the coronary system is the

relevant mechanism for this input–output difference of platelets.

(1) Coronary perfusion pressures were not significantly different between groups before and after application of platelets, thus ruling out large-scale capillary plugging by platelet aggregates. In a previous study, we were able to demonstrate that 10-μm diameter microspheres were almost completely retained (98%) in the capillary system of isolated guinea pig hearts and led to an irreversible increase in coronary resistance, whereas 5-μm diameter microspheres were not retained and did not elevate perfusion pressure [17].

(2) Lamifiban at 1 μM, a concentration that inhibited platelet aggregation via GPIIb/IIIa in-vitro completely (data not shown), did not reduce platelet adhesion to a significant extent. This result is in accordance with studies by others demonstrating that adhesion mechanisms besides those involving GPIIb/IIIa, mainly the interaction of GPIb with von Willebrand's factor, are able to mediate platelet adhesion to the coronary endothelium [19,20].

(3) Electron microscopy of hearts after platelet adhesion revealed adherent platelets in about 5–10% of the observed capillaries, but no aggregate formation or capillary plugging. Location of adherent platelets in the capillary system has also been shown in isolated rat hearts [10].

After coronary passage, platelet glycoproteins GPIIb/IIIa, P-selectin and GPIb each showed a decline of about 10% in their relative immunofluorescence intensity in comparison with the platelets tested before intracoronary application. This uniform decrease is taken as an indication that mainly preactivated platelets with a high expression of glycoproteins became adherent. Activation of platelets during the short time of coronary passage (< 60 s) does not seem to occur, otherwise an increase in GPIIb/IIIa and P-selectin expression would be expected.

Interestingly, adherence of about 1.7×10^7 platelets in the coronary system was sufficient to significantly impair cardiac output after ischaemia and reperfusion (49% recovery vs. 64% without platelets). The reduction of the external heart work in W_2 of the platelet group was mainly due to a dramatic fall of aortic flow of over 50% in comparison to W_1. Coronary flow was only reduced by about 30%, a decline that was also detected in the ischaemia-control group. Some previous studies have described cardioprotective effects of washed platelets of rats. These protective effects were attributed to enhancement of NO production, release of adenosine from platelets and scavenger capabilities of the platelet glutathione redox cycle [10–12], i.e. actions that should lead to coronary dilatation. Moreover, in contrast to our present investigation, the platelets were present before induction of ischaemia and for the entire reperfusion period. In our experiments, platelet infusion occurred only during 1 min of low-flow perfusion, thus making it very unlikely that relevant scavenger effects of platelets could still be exerted in the reperfusion phase. Furthermore, in the studies reporting cardioprotection, the hearts were perfused under normovolaemic conditions (≥ 4 ml/min coronary flow) and then subjected to global ischaemia. Pilot experiments in our group have shown that relevant adherence of washed platelets only occurs under conditions of reduced flow. Rösen et al. [21] demonstrated in an isolated rabbit heart model that the presence of washed human platelets in a situation of coronary artery occlusion prevented recovery of contractility on reperfusion and enlarged the area of infarction due to adhesion of platelets in capillaries near the area at risk. In these experiments, platelets were infused during the period of ischaemia and washed out before the occlusion was released.

Alloatti et al. [8] have reported that perfusion of isolated rabbit hearts with autologous platelets plus platelet-activating factor results in reduction of contractile force. In our system, thrombin was used as stimulating factor to induce thrombogenic properties of the endothelium (externalisation of von Willebrand's factor, expression of platelet-activating factor) [22]. Studies that have demonstrated beneficial effects of platelets did not use any exogenous stimuli. This might be another explanation for the discrepant results.

The application of platelets in the presence of 1 μM lamifiban reduced platelet adhesion to $10.6 \pm 2.3\%$ in comparison with the control group ($16.7 \pm 2.4\%$), which was not statistically significant. Nevertheless, functional recovery of hearts in the platelet plus lamifiban group was significantly better. It should be borne in mind that, in these hearts, about $6–7 \times 10^6$ platelets less became adherent. Assuming a non-linearity between number of adherent platelets and functional infringement or a minimal requirement of adherence, this could be cause enough for improved recovery. In addition, the thromboxane A_2 formation by stimulated platelets is reportedly reduced about 20% by lamifiban [16]. Furthermore, lamifiban itself – applied without platelets – had beneficial effects on the coronary endothelium (reduced transudate formation in the reperfusion phase). Indeed, the parallel infusion of platelets plus lamifiban in the low-flow phase led to the lowest transudate formation in the early reperfusion phase seen in any group (38 ± 8 vs. 71 ± 16 μl/min for platelets alone).

The mechanism by which a small number of adherent platelets infringes myocardial function after ischaemia and reperfusion is intriguing, but remains unclear. Ongoing conversion of the platelet membrane constituent arachidonic acid to cardiodepressants, such as the isoprostanes or thromboxane A_2 [14] presents a likely cause. In animal studies, thromboxane receptor antagonists have been shown to be antiarrhythmic [23] and to reduce infarct size [24].

4.2. Transudate formation

Ischaemia–reperfusion has been shown to cause functional coronary microvascular injury [1], the increase in permeability being proportional to both the duration and

severity of ischaemia. Since myocardial oedema is known to depress contractile function [25], this seemed to be an attractive explanation also for the deleterious action of adherent platelets. Indeed, Kupatt et al. [4] described a progressive increase of transudate formation in isolated guinea pig hearts induced by postischaemic intracoronary application of human granulocytes. The isolated perfused heart model allows a direct measurement of net transmural fluid passage in the entire coronary bed in the form of transudate flow. To our great surprise, the infusion of platelets in the low-flow phase in our experimental system led to significantly decreased – and not increased – formation of transudate in the reperfused hearts, even when reperfusion was extended to 30 min. The application of supernatant of thrombin-stimulated platelets had the identical effect. Thus, degranulation of platelets seems to liberate a long-acting, permeability-reducing principle.

Formation of glycoprotein-rich microparticles after stimulation of washed platelets with thrombin has been demonstrated [26]. Binding of these platelet membrane particles to the coronary endothelium could have elicited a sealing effect. However, we were not able to demonstrate microparticle formation in our experimental setting (data not shown), probably due to the short contact period between thrombin and platelets during coronary passage (20–30 s) and the relatively low thrombin concentration (0.3 U/ml).

For labile substances released by activated platelets, like ADP, adrenalin or serotonin, one would expect a brief effect on vascular permeability. However, adherent platelets could continuously release such agonists. In the platelet group, the correlation coefficient between transudate formation from the 6th to the 10th min of reperfusion and the number of adherent platelets was 0.27 (statistically insignificant). Thus, the transudate-lowering effect seems to be independent of the percentage of adherent platelets. In other words, continuous production of labile factors by these platelets does not seem to invoke the protective effect on the endothelium derived from a bolus of platelets. The long-acting nature of the permeability-reducing effect (still detectable 35 min after application of platelets) drew our attention to protein components known to be liberated from activated platelets, foremost to fibrinogen. According to our results, a single, preischaemic bolus application of fibrinogen, but also one of lamifiban, had the same suppressant effect on transudate formation as did platelets or the supernatant of platelets. Our chosen concentration of 0.6 mg/ml fibrinogen corresponded to about 20% of the level found in human plasma.

With regard to the time course of transudate production in the extended reperfusion phase, all three sealing actions (platelets, fibrinogen, lamifiban) were maintained relative to the ischaemia-controls, but the absolute rate of transudate formation increased with time in all groups. Permeability of water and small hydrophilic solutes is basically influenced by intra- and extravasal hydrostatic and oncotic pressures and by the endothelial barrier. In our model, venous and lymphatic pressure were kept constantly low, as both fluids drained freely from the heart. Oncotic pressure was very low as the perfusion buffer was devoid of proteins. The only variable force was, therefore, the coronary perfusion pressure. In our study, perfusion pressure showed a slight, but continuous increase over the reperfusion phase, without any significant differences between groups at any time of the perfusion protocol. Thus, observed differences of the net fluid transport across the vascular wall at any one time seem to reflect the integrity of the endothelial layer.

The fact that lamifiban reduced transudate formation in the reperfused heart to the same extent as fibrinogen implicates a common mechanism for these two molecularly quite different agents (large peptide vs. small non-peptide) and excludes a purely oncotic action of fibrinogen. Interestingly, both have affinity to receptors recognising the RGD-peptide sequence. Pertinently, in endothelial cell cultures, it has been shown that fibronectin, which also contains an RGD sequence, contributes to the maintenance of the barrier [27]. During application of the fibrinogen bolus as well as during stimulation of platelet secretion thrombin (0.3 U/ml) was present. This might have led to processing of fibrinogen to fibrin. Therefore, the observed suppressant effect on transudate formation could be due to fibrinogen or fibrin actions or both, since both contain the RGD sequence [28]. Cross-linking of fibrin monomers and formation of a fibrin network seems to be unlikely, owing to the lack of factor XIII.

Two adhesion molecules might be of interest in this context: $\alpha_v\beta_3$-integrin (vitronectin receptor) and the intercellular adhesion molecule ICAM-1. For both, the existence on the luminal surface of endothelial cells has been shown [29–32]. Hicks et al. [32] demonstrated on isolated veins that fibrinogen is able to influence vasomotion by binding to ICAM-1. However, the RGD sequence apparently is not involved in the interaction of fibrinogen with ICAM-1 [33,34]. No data are available on fibrin binding to ICAM-1. Nevertheless, D'Souza et al. [35] were able to show that an RGD peptide reduced the binding of fibrinogen to unstimulated ICAM-1, thus suggesting an interaction of RGD sequence containing peptides with the fibrinogen receptor part of ICAM-1. Fibrinogen and fibrin are able to bind to the vitronectin receptor and this binding can be efficiently blocked by RGD-containing peptides [28]. Fibrinopeptides had no influence on the binding of fibrin to the vitronectin receptor. Binding of RGD-containing peptides to the vitronectin receptor of arterioles mediated vasodilation [36]. In view of these multiple actions, fibrinogen, normally present in plasma or in locally high concentrations after degranulation of platelets, or fibrin produced in the presence of thrombin, may partially allay ischaemia–reperfusion injury in the heart by interacting with ICAM-1 or the vitronectin receptor on the luminal surface of coronary endothelial cells.

In conclusion, platelet adhesion to the coronary endothelium in a situation of myocardial ischaemia and reperfusion can further induce cardiac dysfunction. In contrast, degranulation of platelets produced beneficial effects on the integrity of the coronary endothelium in isolated hearts perfused with crystalloid buffer; the active principle most likely being fibrinogen. Due to high plasma fibrinogen already present in-vivo, this endothelial-protective effect of fibrinogen released by platelets may only play a minor role. This would place even more emphasis on the deleterious effects of platelets in vivo.

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