# Activated platelets contribute importantly to myocardial reperfusion injury

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reperfusion injury. *Am J Physiol Heart Circ Physiol* 290: H692–H699, 2006.
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Platelets become activated during myocardial infarction (MI), but the
direct contribution of activated platelets to myocardial reperfusion injury
in vivo has yet to be reported. We tested the hypothesis that activated
platelets contribute importantly to reperfusion injury during MI in mice.
After 30 min of ischemia and 60 min of reperfusion, P-selectin knockout
mice had a significantly smaller infarct size than that of wild-type mice
($P < 0.05$). Platelets were detected by P-selectin antibody in the previ-
ously ischemic region of wild-type mice as early as 2 min postreperfusion
after 45 min, but not 20 min, of ischemia. The appearance of neutrophils
in the heart was delayed when compared with platelets. Flow cytometry
showed that the number of activated platelets more than doubled after 45
min of ischemia when compared with 20 min of ischemia or sham
treatment ($P < 0.05$). Platelet-rich or platelet-poor plasma was then
transfused from either sham-operated or infarcted mice after 45 and 10
min of ischemia-reperfusion to mice undergoing 20 and 60 min of
ischemia-reperfusion. Infarct size was increased by threefold and platelet
accumulation was remarkably enhanced in mice treated with wild-type,
MI-activated platelet-rich plasma but not in mice receiving either platelet-
poor plasma from wild types or MI-activated platelet-rich plasma from
P-selectin knockout mice. In conclusion, circulating platelets become
activated early during reperfusion and their activation depends on the
duration of the preceding coronary occlusion and is proportional to the
extent of myocardial injury. Activated platelets play an important role in
the process of myocardial ischemia-reperfusion injury, and platelet-
derived P-selectin is a critical mediator.

P-selectin; knockout mice

THE INFLAMMATORY RESPONSE elicited by myocardial ischemia-
reperfusion (I/R) injury is associated with enhanced neutrophil
adhesion to endothelial cells and thereafter to reperfused car-
diomyocytes (11, 18, 45). Although most studies of leukocyte
recruitment to postischemic tissues have focused on neutro-
phils, there is growing evidence that other types of blood-borne
cells also contribute importantly to the pathogenesis of I/R
injury. Recent studies undertaken in a variety of organs and
tissues demonstrate that reperfusion of the ischemic heart,
liver, and kidney is associated with the sequestration of circu-
lating T lymphocytes, monocytes, and platelets to these organs,
where they appear to exacerbate the inflammatory response to
I/R injury (3, 5, 25, 33, 34, 38, 46). Leukocytes and platelets
have been found to contribute to I/R injury by interacting with
endothelial cells to promote neutrophil-induced I/R injury (20,
21, 24, 34). Within minutes after reperfusion, platelets are

among the first cells to be recruited and are colocalized with
leukocytes in areas of infarction (7, 25). P-selectin is expressed
on activated platelets and participates in cell adhesion through
its interaction with P-selectin glycoprotein ligand-1 (19, 43).
Activated platelets bind to T lymphocytes, monocytes, and
neutrophils and interact with endothelial cells, thus enhancing
the sequestration of leukocytes in peripheral tissues (15, 20, 21,
27, 35). It is therefore apparent that there exists a temporal and
sequential collaboration between blood-borne cells that ini-
tiates inflammatory responses after I/R injury.

It is well appreciated from clinical and experimental studies
that platelets are activated early during the reperfusion of
ischemic myocardium and that they exacerbate myocardial I/R
injury (20–22). However, the relationships between the dura-
tion of ischemia, postischemic platelet activation, and myocar-
dial infarct size have not been clearly defined in patients or in
animal models in vivo. We hypothesized that platelet activa-
tion early during reperfusion plays a pivotal role in reperfusion
injury after a significant ischemic insult. It followed that infarct
size should be reduced in P-selectin knockout (KO) mice and
that exogenously activated platelets should exacerbate tissue
injury in wild-type hearts subjected to a largely reversible
period of I/R injury. To test this hypothesis, we employed KO
mice and an in vivo mouse model of myocardial I/R injury to
elucidate the role of platelets in mediating reperfusion injury to
the heart after brief or prolonged periods of coronary occlusion
capable of inducing largely reversible or largely irreversible
myocellular damage, respectively.

## METHODS

This study conformed to the *Guide for the Care and Use of
Laboratory Animals* published by the National Institutes of Health
(NIH Publication No. 85-23, Revised 1996) and was conducted under
protocols approved by the Institutional Animal Care and Use Com-
mittee.

*Animals.* One hundred two 8- to 14-wk-old male C57BL/6 (B6) and
congenic P-selectin KO mice were used and assigned to 12 different
groups (Fig. 1). B6 and P-selectin KO mice were purchased from
Jackson Laboratory (Bar Harbor, ME). Nine of these groups were
used for infarct-size determinations and/or immunohistochemistry,
and three groups were used as donors for the platelet-rich and
platelet-poor plasma transfusions. An additional group of B6 mice
($n = 9$) were also included that received 60 min of left anterior
descending coronary artery (LAD) occlusion followed by 60 min of
reperfusion to determine the shortest duration of ischemia that pro-
duced abundant myocellular necrosis.

*Myocardial I/R.* Mice were subjected to 10, 20, 30, 45, or 60 min
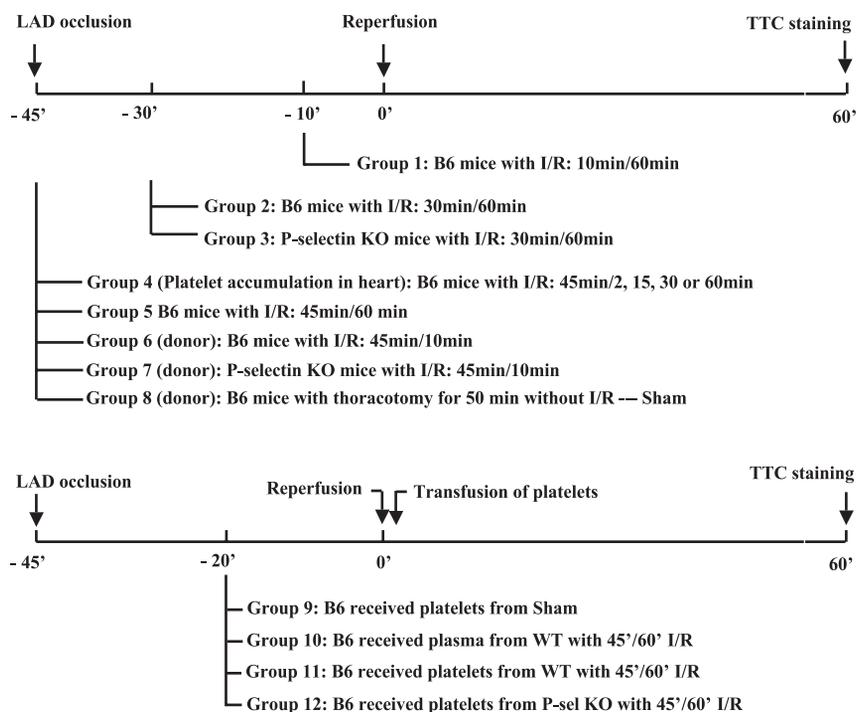of coronary occlusion followed by up to 60 min of reperfusion and

Fig. 1. Experimental protocols for various groups of mice used in this study. LAD, left anterior descending coronary artery; TTC, triphenyltetrazolium chloride; B6, C57BL/6, I/R, ischemia-reperfusion; KO, knockout; WT, wild type; P-sel, P-selectin.

then euthanized to determine myocardial infarct size and to evaluate platelet accumulation in the myocardium. A standard myocardial I/R protocol was employed as detailed previously (44, 45). Briefly, mice were anesthetized with pentobarbital sodium (100 mg/kg ip) and orally intubated. Artificial respiration was maintained at a fraction of inspired oxygen of 0.80 by using 100 strokes/min and a 2- to 3-ml stroke volume delivered through a loose connection from the rodent ventilator. The heart was exposed through a left thoracotomy, and coronary artery occlusion was achieved by passing a suture beneath the LAD and tightening over a piece of polyethylene-60 tubing for a period of from 10 to 60 min. Reperfusion was induced by removing the tubing. Sham-operated mice underwent thoracotomy with a suture placed around the LAD but not tightened. The chest was closed 60 min later. Mice were euthanized either 10 min (as donors) or 60 min (for infarct size determinations) after reperfusion or chest closure.

*MI size measurement.* Mice were euthanized after 60 min of reperfusion, and hearts were cannulated through the ascending aorta for sequential perfusion with 3–4 ml of 1.0% triphenyltetrazolium chloride (TTC) and 10% Phthalo blue. To determine the risk region, the LAD was reoccluded with the same suture used for coronary occlusion in preparation for the Phthalo blue perfusion. The left ventricle (LV) was then cut into five to seven transverse slices that were weighed and digitally photographed for determining infarct size as a percentage of risk region as described previously (44, 45).

*Immunohistochemistry of tissue platelets and neutrophils.* Hearts were harvested and cut into five to seven short-axis slices and immediately fixed in 4% paraformaldehyde in PBS (pH 7.4) for paraffin embedding. Paraffin-embedded sections (5 μm) were rehydrated and incubated with 1% hydrogen peroxide. After being rinsed in PBS, the sections were incubated with 10% blocking serum. Immunostaining was performed with the use of a rabbit polyclonal antibody (1:4,000) against a peptide corresponding to the 25 COOH-terminal amino acids of P-selectin (39)(a gift from Dr. S. A. Green at Univ. of Virginia, Charlottesville). A rat anti-mouse neutrophil antibody (1:1,000) (Serotec, Raleigh, NC) was used to identify tissue neutrophils. The appropriate biotinylated secondary antibodies (Vector Laboratories) were then applied for 1 h at room temperature. After incubation with avidin-biotin complex (Vector Laboratories), immunoreactivity was visualized by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (DAKO) to produce a dark brown precipitate.

*Preparation of platelet-poor plasma and platelet-rich plasma.* Whole blood was acquired by puncturing the right ventricle of fully anesthetized and ventilated donor mice. Blood was immediately anticoagulated by collecting into a one-tenth volume of a solution containing 130 mmol/l of citric acid, 125 mmol/l of trisodium citrate, and 110 mmol/l of glucose with immediate mixing on collection of the necessary volume. Platelet-poor plasma was acquired by centrifuging the whole blood at 2,000 $g$ for 30 min (22°C). Platelet-rich plasma was acquired by centrifuging the whole blood at low speed (100 $g$, 8 min, 22°C). Both platelet-poor plasma and platelet-rich plasma were used within 1 h after preparation. Cell counts of platelets, white blood cells (WBC), and red blood cells (RBC) in platelet-poor or platelet-rich plasma were performed with a HemaVet Hematology System (CDC Technologies, Oxford, CT).

*Flow cytometry to evaluate activated platelets.* Platelet-rich plasma samples acquired from sham-operated mice or after (20 or 45 min) LAD occlusion followed by 10 min of reperfusion were coincubated for 30 min with phycoerythrin-conjugated anti-CD31 (to label total platelets) and with FITC-conjugated anti-P-selectin (to label activated platelets). Analysis by flow cytometry was then conducted on a FACScan instrument (Becton Dickinson, Palo Alto, CA). The hearts from these same mice were processed for immunohistochemistry.

*Dose of plasma or platelets delivered to recipient mice.* One donor mouse was used to transfuse three to four recipients, which received intravenous transfusion of either platelet-poor or platelet-rich plasma immediately after the initiation of reperfusion after 20 min of ischemia through the external jugular vein. The dose of platelet-poor plasma was 60 μl/mouse. The dose of platelet-rich plasma was adjusted as necessary to deliver $2 \times 10^7$ platelets/mouse in a volume of 30–60 μl.

*Statistical analysis.* Data were reported as means ± SE. Infarct sizes, risk region sizes, and flow cytometry results were compared with one-way ANOVA followed by Student's $t$-tests for unpaired data with the Bonferroni correction. The nonparametric Kruskal-Wallis test was used for comparisons of infarction and risk region among multiple groups. To determine which pairs of multiple groups were

different, the Dunn multiple comparisons adjustment was applied to the Kruskal-Wallis test.

## RESULTS

A total of 111 mice were used in this study. Two mice died immediately after reperfusion, and two mice were excluded because of technical difficulties in restoring reperfusion (as indicated by the absence of color changes in the ischemic region on removal of the coronary ligation). Among the excluded mice, one received transfusion of platelet-poor plasma and the other received transfusion of platelet-rich plasma.

*Myocardial infarct size after variable periods of LAD occlusion.* For the purposes of the current study, it became necessary to identify not only the longest period of ischemia that would result in minimal myocardial necrosis but also the shortest period of ischemia that would result in abundant myocardial necrosis. To study the myocardial infarct size as a function of occlusion time, five groups of wild-type B6 mice underwent 10, 20, 30, 45, or 60 min of LAD occlusion, respectively, followed by a fixed 60 min of reperfusion. The risk region (percentage of LV mass) was not significantly different among the five groups; however, infarct size increased significantly with the duration of ischemia (Table 1 and Fig. 2). Infarct size was minimal after 20 min of LAD occlusion; however, it increased from $6.8 \pm 2.9$ to $37.4 \pm 3.9$ (percentage of risk region) between 20 and 30 min of LAD occlusion (fivefold increase; $P < 0.05$). Infarct size continued its dramatic increase as LAD occlusion time was increased from 30 to 45 min, growing from $37.4 \pm 3.9$ to encompass $57.8 \pm 3.1\%$ of the risk region (1.5-fold increase; $P < 0.05$). Between 45 and 60 min of LAD occlusion, infarct size only increased from $57.8 \pm 3.1$ to $65.3 \pm 2.4\%$ of the risk region, a difference that failed to reach statistical significance [$P =$ not significant (NS)] (Table 1 and Fig. 2). Thus, in B6 mice, a brief 20-min period of LAD occlusion induces an extremely small infarct, whereas infarct size grows quickly over the next 25 min and reaches a plateau at 45 min such that the increase in infarct size between 45 and 60 min of LAD occlusion is essentially negligible. On the basis of this time course study of myocardial infarction (MI) in B6 mice, the following experimental design employed a 30-min LAD occlusion protocol to determine the role of P-selectin in mediating myocardial I/R injury in B6 and KO mice, a 45-min protocol to acquire MI-activated platelet-poor and platelet-rich plasma, and a 20-min protocol to demonstrate the contribution of activated platelets to reperfusion injury in wild-type mice.

*Significant role of P-selectin in mediating MI.* Myocardial infarct size was evaluated in wild-type B6 mice and congenic P-selectin KO mice after 30 min of LAD occlusion and 60 min
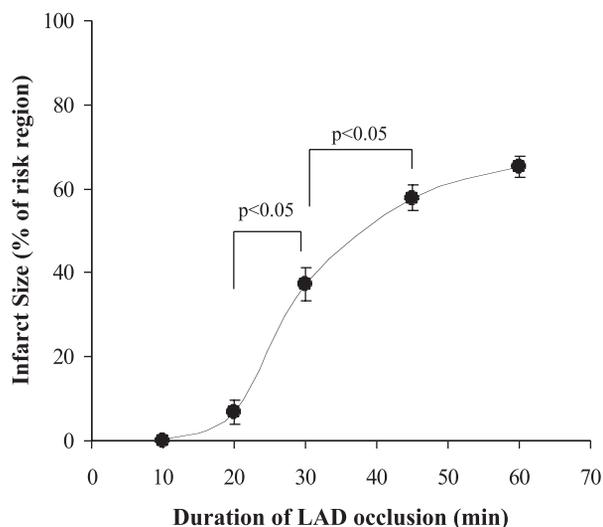


Fig. 2. Infarct size as a function of duration of coronary occlusion in mice. Changes in myocardial infarct size are plotted against duration of LAD occlusion followed by a fixed (60 min) period of reperfusion. Increase in infarct size with increasing duration of occlusion follows a sigmoidal curve with a rapid increase in infarct size occurring between 20 and 45 min of LAD occlusion.

of reperfusion. There was no difference in risk region as a percentage of LV mass between the two groups ($42.2 \pm 1.3$ vs. $43.2 \pm 1.5\%$; $P =$ NS). Infarct size (percentage of risk region) was significantly smaller in P-selectin KO than in wild-type mice ($22.8 \pm 2.4$ vs. $37.4 \pm 3.9\%$; $P < 0.05$) (Fig. 3).

*Recruitment of platelets and neutrophils in myocardium after prolonged ischemia.* Essentially no platelets or neutrophils were detected by immunohistochemistry in the myocardial tissue of sham-operated B6 mice. After 45 min of LAD occlusion, platelets were found lined up and/or clotted in the vasculature of previously ischemic myocardium as early as 2 min after the initiation of reperfusion but not in nonischemic myocardium. The degree of platelet recruitment continued to increase in the vasculature and interstitial myocardium over time up to 60 min postreperfusion (Fig. 4, *top*). Although neutrophils did appear in the previously ischemic myocardium early after reperfusion, they accumulated much more slowly than platelets and only became remarkable after 30 min of reperfusion (Fig. 4, *bottom*).

*Relationship of platelet activation with duration of myocardial ischemia.* Expression of membrane P-selectin was used to quantitatively determine the activation of circulating platelets by flow cytometry. In B6 mice with a sham operation or after 20 min of LAD occlusion, 2–2.2% of all circulating platelets

Table 1. *Infarct size as a function of ischemia in mice*

| | Duration of LAD Occlusion, min | | | | |
|---|---|---|---|---|---|
| | 10 | 20 | 30 | 45 | 60 |
| Risk region, | | | | | |
| % LV | $40.3 \pm 2.9$ | $38.1 \pm 1.9$ | $43.2 \pm 1.5$ | $41.8 \pm 1.5$ | $42.9 \pm 3.1$ |
| Infarct size, | | | | | |
| % RR | 0 | $6.8 \pm 2.9$ | $37.4 \pm 3.9$ | $57.8 \pm 3.1*$ | $65.3 \pm 2.4*$ |
| % LV | 0 | $2.4 \pm 1.0$ | $16.1 \pm 1.8$ | $24.3 \pm 1.8*$ | $28.0 \pm 2.1*$ |

Values are means $\pm$ SE; $n = 9$ mice. LAD, left anterior descending coronary artery; LV, left ventricle; *$P < 0.05$ vs. 10, 20, and 30 min of occlusion.
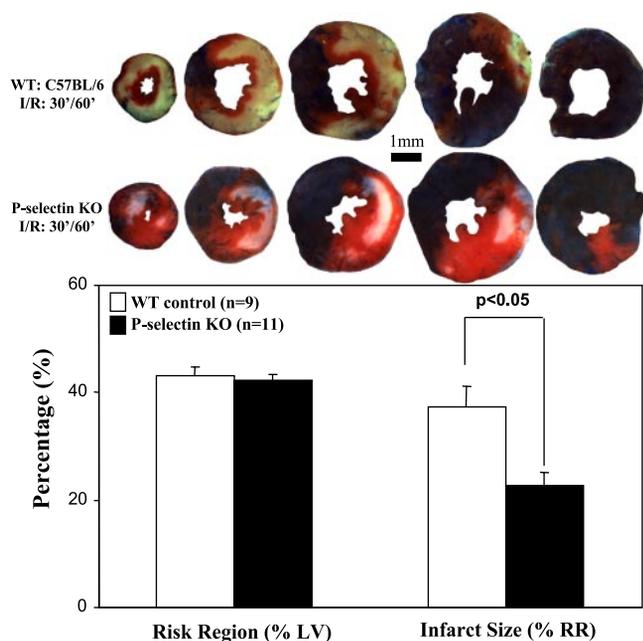
Fig. 3. Myocardial infarct size in WT and congenic P-selectin KO mice. *Top*: myocardial infarct size measured by TTC-Phthalo Blue staining after 30 and 60 min of I/R in WT mice and P-selectin KO mice. *Bottom*: myocardial infarct size [as percentage of risk region (RR)] was significantly smaller in P-selectin KO mice than in WT mice ($22.8 \pm 2.4$ vs. $37.4 \pm 3.9\%$; $P < 0.05$). LV, left ventricle.

were found to be activated ($P = $ NS). However, platelet activation more than doubled in B6 mice after 45 min of LAD occlusion ($4.49 \pm 0.05\%$ compared with sham-operated mice or after 20 min of ischemia; $P < 0.05$; Fig. 5, *top*). Tissue sections showed no evidence or only a few platelets accumulated in myocardium from sham-operated mice or from mice subjected to 20 min of ischemia. In contrast, platelet accumulation was abundant in tissue sections from mice subjected to 45 min of ischemia (Fig. 5, *bottom*).

*Exogenously activated platelets exacerbate MI in mice subjected to brief ischemia.* Exogenous platelet-poor/rich plasma was acquired from wild-type B6 or P-selectin KO mice undergoing either sham operation or 45 min of ischemia and 10 min of reperfusion (MI activated). The platelet-poor plasma was completely free of blood corpuscles as detected by the HemaVet Hematology System. In platelet-rich plasma, the concentration of platelets was $4.43 \pm 0.78 \times 10^5/\mu l$, whereas the concentration of both WBC and RBC was negligible (WBC, $60 \pm 10/\mu l$; RBC, $10 \pm 5 \times 10^3/\mu l$). The recipient B6 mice underwent 20 min of LAD occlusion and 60 min of reperfusion. There was no difference in risk region among the four recipient groups. In mice that received transfusions of either platelet-rich plasma from sham-operated donors or platelet-poor plasma from MI-activated donors, the infarct size was $6.8 \pm 2.9$ and $9.9 \pm 3.8$ (percentage of risk region), respectively ($P = $ NS; Fig. 6). In mice treated with platelet-rich plasma from wild-type MI-activated donors, infarct size was significantly increased four- to fivefold to $34.8 \pm 2.6\%$ (compared with any other recipient group; $P < 0.05$). Interestingly, infarct size in wild-type mice subjected to a 20-min LAD occlusion (and treated with platelet-rich plasma from wild-type MI-activated donors; Fig. 6) was similar to that found in

wild-type mice subjected to a 30-min LAD occlusion ($34.8 \pm 2.6$ vs. $37.4 \pm 3.9$; $P = $ NS; Fig. 3). However, in mice treated with platelet-rich plasma from P-selectin KO MI-activated donors, infarct size was only slightly increased and not significantly different from that of mice receiving either platelet-rich plasma from sham-operated mice or platelet-poor plasma from wild-type MI-activated donors (Fig. 6, *middle* and *top*). Immunohistochemistry detected significantly more myocardial platelet accumulation in recipient mice treated with wild-type MI-activated platelets (Fig. 6, *bottom*).

## DISCUSSION

The current study clearly demonstrates the sigmoidal relationship between myocardial infarct size and duration of ischemia, wherein a brief 20-min LAD occlusion induces extremely small infarctions, whereas a more prolonged 45-min LAD occlusion induces infarcts nearly as large as those induced by a 60-min LAD occlusion. With a 30-min LAD occlusion, infarct size was significantly smaller in P-selectin KO mice than in B6 mice, which indicates that P-selectin contributes to myocardial I/R injury. Using P-selectin as marker, we found that in wild-type mice, circulating platelets are activated early during reperfusion and their activation is dependent on the duration of the preceding coronary occlusion. Platelets were recruited into previously ischemic myocardium as early as 2 min after reperfusion. The accumulation of platelets in the myocardium is greatly accelerated compared with that of neutrophils, which do not become remarkable until 30 min postreperfusion. Thus platelets are among the first blood cells to adhere to the vasculature within previously ischemic myocardium during reperfusion and contribute importantly to the postreperfusion no-reflow phenomenon and cardiac inflammation. Transfusion of activated platelets, acquired at early reperfusion from mice with prolonged ischemic insult, significantly
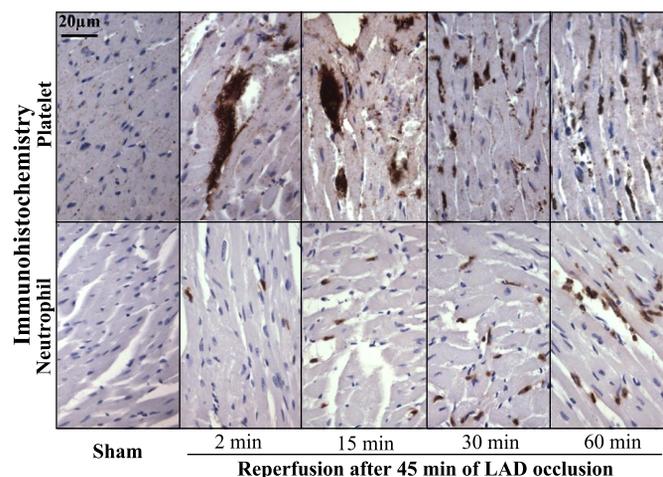


Fig. 4. Time course of platelet deposition and neutrophil recruitment into previously ischemic myocardium. Immunohistochemistry (IHC) was used to assess ischemic myocardium at regular time points during the first 60 min of reperfusion after 45 min of LAD occlusion in B6 mice. *Top*: platelets are stained golden-brown by the P-selectin antibody. P-selectins expressed by endothelial cells are not detectable. Platelet accumulation and clot formation are readily evident from as early as 2 min continuing on through 60 min postreperfusion. *Bottom*: neutrophils, stained golden-brown by anti-neutrophil antibody, can barely be detected in myocardium at 2 min postreperfusion and gradually increase in number over the first 60 min postreperfusion.

Fig. 5. Flow cytometry and IHC of platelet activation. *Top*: results of flow cytometry. Total platelets were detected by anti-CD31, and activated platelets were detected by anti-P-selectin. Approximately 2% of circulating platelets were activated in mice undergoing sham operation or 20 min of ischemia. However, percentage of activated platelets more than doubled in mice with 45 min of ischemia ($P < 0.05$ vs. other groups). *Bottom*: in agreement with flow cytometry results, myocardial P-selectin expression was significantly enhanced in mice after 45 min of ischemia but not after 20 min of ischemia.
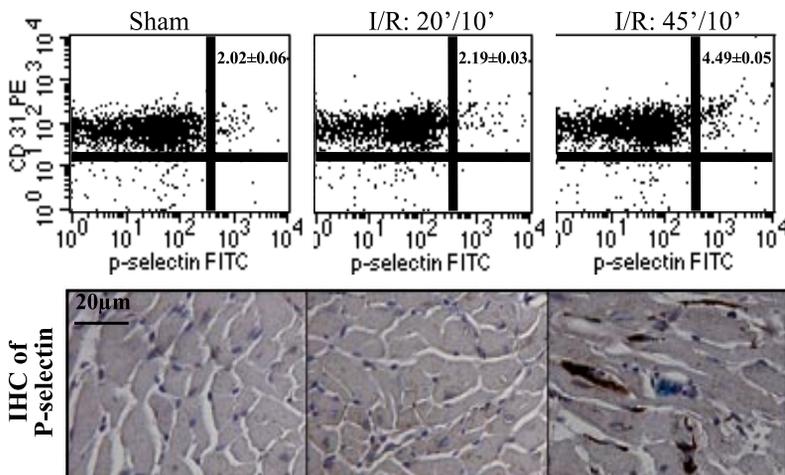
exacerbates MI in mice subjected to a brief period of coronary occlusion that would normally induce a minimum of infarction. Recent studies undertaken in other organ systems further support the contention that the activated platelets contribute importantly to reperfusion injury (7, 20, 24, 25, 34). The studies presented here unequivocally establish that platelet-derived P-selectin plays a critical role in mediating myocardial reperfusion injury in mice.

*Myocardial I/R injury in mice and role of P-selectin in mediating I/R injury.* Mouse models of MI have been employed extensively in modern cardiovascular research because of the widespread availability of transgenic and KO mice. Different periods of coronary occlusion have been employed by different researchers in their efforts to define the underlying mechanisms or develop new therapies (17, 44, 45). There is a

general understanding that infarct size studies should be conducted in experimental animal models by using an ischemic period that produces a moderately sized infarct (25–50%) so that there is room for a treatment to increase or decrease infarct size. By using 30 or 60 min of LAD occlusion in mice, Palazzo et al. (31) reported that infarct size was significantly reduced in P-selectin KO mice after 30-min occlusion but not after 60-min occlusion. However, the sigmoid relationship between the period of coronary occlusion and the resulting infarct size has not been clearly defined in previous studies. In the current study, five groups of wild-type B6 mice underwent five different periods of LAD occlusion followed by 60 min of reperfusion. We found that myocardial infarct size increases as a sigmoidal function of ischemic duration, with little infarction occurring during the first 20 min and a steep increase in infarct



Fig. 6. Exogenously activated platelets aggravate myocardial infarction. *Top* and *middle*: myocardial infarct size as measured by TTC and Phthalo blue staining after 20 and 60 min of I/R in WT mice (C57BL/6) treated with platelet-rich plasma from sham-operated mice, platelet-poor plasma, or activated platelet-rich plasma isolated from freshly infarcted WT and P-selectin KO mice. Infarct size was increased up to fourfold in group treated with WT myocardial infarction (MI)-activated platelet-rich plasma. Mice treated with P-selectin KO MI-activated platelet-rich plasma had a smaller mean infarct size than that of those treated with WT MI-activated platelet-rich plasma ($P < 0.05$). *Bottom*: immunostaining of previously ischemic myocardium revealed an increase in P-selectin expression in mice that were treated with MI-activated platelets.
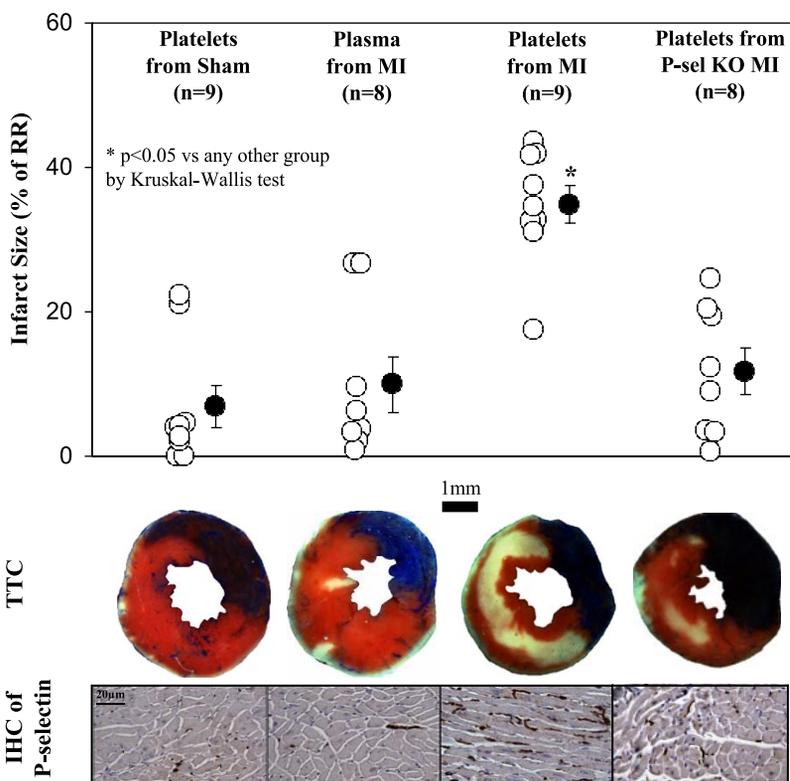
size between 20 and 45 min, followed by a minimal increase in infarct size between 45 and 60 min (Table 1 and Fig. 1). The results indicate that LAD occlusion for a duration of 30 to 45 min will induce a relatively large infarct size and should be suitable for studies in mice aimed at reducing or enhancing MI. On the basis of this result, we employed a 30-min LAD occlusion model to define the role of P-selectin in the process of myocardial I/R injury. Similar to the result reported by Palazzo et al. (31), the current study showed that myocardial infarct size was significantly reduced in P-selectin KO mice by up to 40% when compared with B6 mice, indicating that P-selectin plays an important role in mediating myocardial I/R injury (Fig. 3). Immunostaining of myocardial platelet deposition in the various groups revealed that P-selectin was abundantly expressed on platelets found clumped in reperfused territories; however, endothelial P-selectin was not detected (Figs. 4–6), suggesting that platelets, or platelet-derived P-selectin, may be critical in mediating myocardial I/R injury. In the P-selectin KO mice, the number of circulating platelets was similar to the wild-type control mice ($6.1 \times 10^5$ vs. $5.2 \times 10^5$ platelets); however, bleeding time was significantly prolonged (1.9 vs. 1.5 min; $P < 0.05$; see Table 2). Similar results have previously been reported, and the prolonged bleeding time in P-selectin KO mice may be secondary to compromised platelet rolling (9). The reduction in platelet rolling may be one of the reasons for reduced inflammatory responses after MI in P-selectin KO mice. Interestingly, we found that WBC were significantly increased in P-selectin KO mice (four times greater than that of wild-type mice; $P < 0.05$; Table 2). The disassociation between the peripheral leukophilia and the reduced inflammatory response after myocardial I/R injury in P-selectin KO mice further indicates that P-selectin plays a critical role in mediating leukocyte recruitment during inflammatory response (16).

Whereas our result showing that infarct size is reduced in P-selectin KO mice is entirely compatible with Palazzo et al. (31), it may appear incongruent with work from Briaud et al. (4) showing that mice with a deficiency in both ICAM-1 and P-selectin had a similar myocardial infarct size as wild-type control mice after receiving either 30 or 60 min of ischemia. However, differences in experimental design (single vs. double KO) as well as methodological differences (1-h vs. 24-h follow-up, respectively) preclude direct comparison between the current study and this previous report (4).

*Platelets are activated during postischemic reperfusion.* There is an increasing body of evidence showing that platelets are activated during ischemia and during early reperfusion after ischemia. Platelet activation pathways involve thrombin, collagen, reactive oxygen species, cytokines (e.g., IL-1β, TNF-α, and IFN-γ), complement, and epinephrine (6, 20, 23, 29, 30, 40, 41). During the acute phase of unstable angina, patients exhibit increased coagulation system activity and develop frequent bursts of thrombin production (2, 26). Animal experiments in a model of mesentery I/R demonstrate that antithrombin III prevents and rapidly reverses leukocyte recruitment (29). The tissue factor-driven accumulation of fibrinogen has been reported during reperfusion of ischemic organs, and the deposition of fibrinogen colocalizes with large numbers of adherent platelets (20, 25, 28). This fibrinogen-dependent platelet activation plays a key role in the development of I/R-induced inflammatory responses (25). In a rat model of myocardial I/R injury, upregulation of IL-1-β, TNF-α, and IFN-γ in myocardial tissue was significantly enhanced after 30 min of LAD occlusion and 60 min of reperfusion (13). IL-1-β, TNF-α, and IFN-γ are reportedly strong activators of platelets (32, 41, 42). Using in vivo models of I/R in mice, the current study clearly demonstrates that platelets are activated early during reperfusion after myocardial ischemia. We found that circulating platelets are activated to some extent by the invasive surgical procedures necessary to create I/R injury, but platelet activation is increased significantly by the processes of myocardial I/R. Furthermore, the degree of platelet activation correlates with the duration of ischemia and the amount of tissue necrosis. Thus, when assessed 10 min after reperfusion, mice receiving 45 min of ischemia produced twice the number of activated circulating platelets when compared with those receiving 20 min of ischemia (Fig. 5). The molecular mechanisms that lead to platelet activation during myocardial I/R were not directly investigated in the current study, but they may represent important targets for developing novel therapeutic interventions against reperfusion injury.

*Activated platelets mediate myocardial I/R injury.* There is solid proof that unstable angina and acute MI are associated with increased platelet aggregability and an increased proportion of activated platelets expressing P-selectin in blood (1, 27, 35). The magnitude of these changes has been consistently found to correlate with the severity of the clinical manifestation of the acute coronary syndrome (1, 10). Although limited, several animal experiments have shown that myocardial stunning after brief coronary occlusion is associated with substantial dynamic changes in platelet aggregability and other hemostatic factors (37). For example, inhibiting the activity of platelets, but not of neutrophils, improves contractile recovery from myocardial stunning (14). Furthermore, the addition of platelets to the perfusate impairs coronary blood flow (22), reduces postischemic contractile recovery (12, 36), and promotes arrhythmia (8) in numerous models of myocardial I/R injury. Prolonged ischemia followed by reperfusion thus induces profound inflammatory responses, which not only contribute to myocardial contractile dysfunction but also exacerbate myocardial tissue injury. However, the direct contribution of activated platelets to myocardial injury after prolonged

Table 2. *Bleeding time and blood cell count*

| Mice | BT, min | Hb, g/dl | Hct, % | WBC, $10^3/\mu l$ | NE, $10^3/\mu l$ | LY, $10^3/\mu l$ | Plt, $10^3/\mu l$ |
|---|---|---|---|---|---|---|---|
| Wild type | 1.9±0.1 | 10.8±1.4 | 38±4 | 2.8±0.8 | 0.6±0.2 | 2.0±0.5 | 611±48 |
| P-sel KO | 1.5±0.1 | 13.1±0.2 | 45±2 | 13.3±0.8 | 3.5±0.2 | 9.5±0.7 | 523±46 |
| P value | <0.05 | NS | NS | <0.05 | <0.05 | <0.05 | NS |

Values are means ± SE; $n = 5$ mice/group. BT, bleeding time; Hb, hemoglobin; Hct, hematocrit; WBC, white blood cells; NE, neutrophils; LY, lymphocytes; Plt, platelets; P-sel, P-selectin; KO, knockout; NS, not significant.

periods of ischemia has not been previously reported. The current study for the first time shows that circulating platelets are activated in an injury-dependent manner during reperfusion after myocardial ischemia. Platelets were found in the myocardium immediately on reperfusion. Immunohistochemisty identified platelets lining the vascular endothelium and aggregating as clots in the microvasculature. Platelet deposition was evident as early as 2 min postreperfusion and was confined exclusively to the previously ischemic myocardium, not to the nonischemic region. Platelet deposition became more extensive over time up to 60 min after reperfusion. Neutrophils were found to be recruited to the same ischemic regions as were platelets, but neutrophil infiltration was delayed in comparison to platelet deposition (Fig. 4). The fact that activated platelets were found only in the previously ischemic myocardium suggests that endothelial activation or dysfunction and/or cytokines or chemokines released in the ischemic myocardium contribute to the platelet deposition. Although the current study provides no direct evidence to show that P-selectin is involved in platelet deposition, it likely plays an important role in this process because it also contributes to I/R injury (Figs. 3 and 6). The degree of platelet activation in the bloodstream is proportional not only to the duration of the ischemic insult but also to the degree of myocardial platelet deposition and final infarct size (Figs. 2 and 5). Although the percentage of activated platelets in the bloodstream did not appear to be tightly correlated with infarct size, it should be noted that the percentage of circulating activated platelets does not include those platelets that have already been deposited in the ischemic myocardium (Figs. 4 and 5). To confirm the role of activated platelets in myocardial I/R injury, we studied the ability of exogenously activated platelets to exacerbate infarct size in mice undergoing minimally lethal ischemic episodes. The exogenously activated platelets (obtained from mice after 45 min of myocardial ischemia and 10 min of reperfusion) were delivered to the mice on reperfusion after 20 min of ischemia and were found to significantly enhance platelet deposition in the myocardium and to increase infarct size by threefold (Fig. 6). By using P-selectin KO mice and platelets deficient in P-selectin, we went on to show that platelet-derived P-selectin plays a critical role in the process of platelet-mediated myocardial I/R injury.

In summary, circulating platelets become activated early during reperfusion and their activation depends on the duration of the preceding coronary occlusion. Activated platelets are deposited in the previously ischemic myocardium proportional to the extent of myocardial injury. Transfusion of activated platelets, acquired at reperfusion from mice subjected to a prolonged ischemic insult, markedly exacerbates MI in mice subjected to a brief coronary occlusion that would normally induce a minimum of infarction. Taken together, these results show that activated platelets play an important role in the process of myocardial I/R injury and that platelet-derived P-selectin is a critical mediator.

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