Inhibition of Intravascular Platelet Aggregation by Endothelium-Derived Relaxing Factor: Reversal by Red Blood Cells

By Donald S. Houston, Pauline Robinson, and Jon M. Gerrard

Studies were performed to determine whether endothelium-derived relaxing factor (EDRF) can inhibit platelet aggregation within the vascular lumen, and if so, whether the inhibition persists in the presence of red blood cells (RBCs). Canine femoral arteries mounted in an organ bath were perfused with physiologic saline solution to which acetylsalicylic acid was added to block prostacyclin formation. During contraction with phenylephrine, addition of acetylcholine to the perfusing solution to evoke EDRF release relaxed the vessel wall. Washed human platelets labeled with $^{14}$C-5-hydroxytryptamine were added to the perfusing solution, and activated by thrombin infused via a branch vessel. The perfusate was collected downstream and centrifuged; the fraction of $^{14}$C-5-hydroxytryptamine appearing in the supernatant reflected the degree of platelet activation. Stimulation of EDRF release with acetylcholine inhibited $^{14}$C-5-hydroxytryptamine release. Hemoglobin (Hb) (10⁻⁶ mol/L) blocked vascular relaxation and platelet-inhibition. RBCs at a hematocrit of 10% (treated with echotothiophate to block erythrocyte cholinesterase) did not prevent relaxation but reversed the platelet inhibition. Lower hematocrits did not completely block the inhibition. Thus, erythrocyte Hb may modulate the inhibition of intraluminal platelet aggregation by EDRF.

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ENDOTHELIAL CELLS can release a nonprostanoid vasodilator, known as endothelium-derived relaxing factor (EDRF), in response to a variety of stimuli such as acetylsalicylic acid and bradykinin.¹ ² A product of endothelial cells, seemingly identical to EDRF, can also inhibit the aggregation of platelets, as studied in vitro.³ ⁴ Whether EDRF inhibits platelets in vivo is less certain. Free hemoglobin (Hb) can inhibit endothelium-dependent relaxation, apparently by binding EDRF.⁵ Hb contained within red blood cells (RBCs) might also bind EDRF. Therefore, the current experiments were undertaken to determine whether EDRF released intraluminally in a perfused artery can inhibit activation of platelets flowing through that vessel, and whether RBCs can interfere with this inhibitory effect.

MATERIALS AND METHODS

Tissue preparation. Femoral arteries were obtained from mongrel dogs of either sex, 15 to 30 kg, anesthetized with sodium pentobarbital (30 mg/kg intravenously [IV]), given heparin 5,000 U IV, and exsanguinated. The entire vessel from abdominal wall to popliteal region was removed carefully. The artery was placed in cold physiologic saline solution (composition below) and cleaned of loose adherent connective tissue. One side branch was selected about 2 cm from the proximal end, and was cannulated for infusion of thrombin. All remaining side branches were ligated. At a branch-free site in the distal half of the vessel, two fine wire hooks were pierced through the vessel wall on opposite sides (see Fig 1).

The vessel was placed in a chamber containing 600 mL of physiologic saline solution, maintained at 37°C by immersion in a heating bath and gassed with 10% O₂/5% CO₂/85% N₂. Each end of the vessel was cannulated; the proximal end was connected to a roller pump that perfused the vessel at a rate of 2 mL/min. The roller pump drew from a reservoir of physiologic saline solution with 1% albumin. Each end of the vessel was connected to a roller pump that perfused the vessel at a rate of 2 mL/min. The roller pump drew from a reservoir of physiologic saline solution with 1% albumin. Each end of the vessel was connected to a roller pump that perfused the vessel at a rate of 2 mL/min. The roller pump drew from a reservoir of physiologic saline solution with 1% albumin.

One of the hooks placed through the vessel wall was connected to a clip anchored on the bottom of the chamber, while the other was connected to a force transducer (model UTC3; Gould Inc, Cleveland, OH) for measurement of isometric tension. At the beginning of the experiment the vessel was stretched gradually to a resting tension of 4 g.

Platelet preparation. Blood was obtained by venupuncture from healthy volunteers who had taken no aspirin or other drugs within the preceding week. Blood, 48.6 mL, was collected into 11.4 mL of citrate anticoagulant, and centrifuged at 200g for 25 minutes at room temperature. The platelet-rich plasma thus obtained was incubated with $^{14}$C-5-hydroxytryptamine (740 Bq/mL) for 30 minutes, then centrifuged at 800g for 10 minutes. The pellet thus obtained was resuspended in wash solution (composition below) to a volume of 25 mL. This platelet suspension was then centrifuged at 800g for 30 seconds to sediment out contaminating RBCs and white blood cells, and the platelet suspension decanted. Just before use, aliquots of this platelet suspension were taken, one-fifth volume of citrate anticoagulant added, and then centrifuged at 800g for 10 minutes; the pellet thus obtained was resuspended in 4 mL of the perfusing solution drawn from the reservoir chamber currently supplying the vessel (ie, gassed and maintained at 37°C, and containing drugs, Hb, or RBCs).

RBC preparation. The packed RBCs obtained as above were suspended in 3 vol of wash solution. The acetylcholinesterase inhibitor, echothiophate; was added to a final concentration of 10⁻⁶ mol/L and incubated for 30 minutes. The cells were then centrifuged at 800g for 10 minutes, and washed by resuspension in wash solution and repeat centrifugation. The packed cells thus obtained were diluted to the desired hematocrit in physiologic saline solution with 1 g/L albumin.

Drugs and solutions. Except as noted, drugs were dissolved in distilled water and diluted in physiologic saline to the appropriate concentrations. Acetylcholine chloride, acetylsalicylic acid (dissolved in 50 mmol/L Tris HCl), bovine albumin (fatty acid free), citrate anticoagulant, and 10% CO₂/85% N₂, each were dissolved in 10 mmol/L sodium acetate buffer (pH 8.5) to obtain a 10⁻⁶ mol/L stock solution that was adjusted with 0.1 N NaOH to bring the pH to 7.4.

From the Departments of Medicine and Pediatrics, University of Manitoba, Winnipeg, Canada.

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Address reprint requests to Jon M. Gerrard, MD, PhD, Manitoba Institute of Cell Biology, 100 Oliva St, Winnipeg, Manitoba R3E 0V9, Canada.

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bovine Hb, and phenylephrine hydrochloride were obtained from Sigma Chemical Co (St Louis, MO). Bovine thrombin (Thrombostat) was obtained from Parke-Davis (Scarborough, Ontario, Canada). Echotriphosphate iodide (Ayerst) was dissolved in the supplied diluent and stored refrigerated in this stock solution. [2-3H]-5-hydroxytryptamine binoxalate (2.2 GBq/mmol; 3.7 MBq/mL) was obtained from New England Nuclear (Boston MA).

Bovine Hb (which is largely in the oxidized or methemoglobin form) was prepared in the reduced form after the method of Martin et al. A 10^{-3} mol/L solution of Hb was prepared fresh daily in 5 mL distilled water, and a 40-fold molar excess of sodium dithionite (Na,S,O_2) added. To remove the dithionite, the solution was dialyzed in 2 L distilled water with 0.01% EDTA at 4°C for 2 hours. For 30 minutes before and throughout the dialysis, the water was gassed with N_2. The content of reduced Hb thus obtained (55% to 80% of the total) was determined indirectly by spectrophotometry (Beckman DU-8) at 632 nm. The amount of Hb used was adjusted accordingly.

Physiologic saline solution contained the following (except noted, all concentrations given in millimolar): NaCl 118.3, KCl 4.7, CaCl_2 1.2, MgSO_4 1.2, KH.PO_4 1.2, NaHCO_3 25, Na_2EDTA 0.026, and glucose 11.1; pH was adjusted to 7.4 after gassing with 5% CO_2.

When used for perfusion, 1 g/L bovine albumin was added. Wash solution (modified Hanks' balanced salt solution) contained: NaCl 137, KCl 5.36, KH.PO_4 0.441, Na,HPO_4 0.177, NaHCO_3 3.35, MgCl_2 0.5, glucose 10, and 1 g/L bovine albumin; pH was adjusted to 7.35. Citrate anticoagulant contained: trisodium citrate 85, citric acid 65, glucose 111. Glutaraldehyde was diluted to 0.1% (vol/vol) in White's saline (NaCl 120, KCl 5, MgSO_4 2.3, Ca [NO_3_2] 3.2, NaHCO_3 6.5, Na,HPO_4 0.41, KH.PO_4 0.19, Phenol Red 0.014; pH 7.4).

Aggregometry studies. Platelets prepared as above and suspended in physiologic saline solution with 1 g/L bovine albumin were studied in a light-transmission aggregometer (Payton Associates, Scarborough, Ontario, Canada). Aggregometry was performed at 37°C in 1-mL siliconized glass cuvettes, with magnetic stirring of the platelets. The baseline of the recording pen was set using the platelet suspension, and 100% aggregation was set using the physiologic saline solution. Light transmission was measured and percent aggregation recorded at 2 minutes after addition of bovine thrombin (0.25 U/mL). Aggregation was stopped 3 minutes after addition of the agonist by addition of an equal volume of 0.1% glutaraldehyde.

In cases where RBCs were incorporated in the suspension, the volume of physiologic saline solution was decreased accordingly so that the number of platelets was constant. Because of their opacity, when RBCs were present light transmission could not be recorded.

After aggregation was stopped, the samples were centrifuged at 800g for 10 minutes. Supernatant, 0.5 mL, was removed and 3.0 mL of scintillation fluid (Ecolite, ICN, Irvine, CA) added for scintillation counting. The pellet was washed in 0.5 mL Tris HCl with 1% sodium dodecyl sulphate and counted in the same way. 5-Hydroxytryptamine release was expressed as the number of counts in the supernatant divided by the total counts (pellet plus supernatant). In cases where the pellet contained RBCs, it was resuspended in 20 mL of physiologic saline solution to dilute the RBCs, which would otherwise interfere with the scintillation counting. An aliquot of 0.5 mL of this suspension was taken for counting; the total counts thus obtained were only slightly less than those obtained when no RBCs were included, indicating that there was no substantial quenching due to the RBCs.

Perfusion studies. After mounting in the tissue chamber, the arteries were perfused with physiologic saline solution with 1 g/L bovine albumin. Acetylsalicylic acid (10^{-4} mol/L) was added to the perfusion fluid for the initial 30 minutes to block endothelial production of prostacyclin. The vessels were then contracted by adding phenylephrine (3 x 10^{-4} to 10^{-3} mol/L) to the bath solution surrounding the vessel; the vessel remained in contact with phenylephrine for the remainder of the experiment. After a stable contraction had been obtained, presence of functional endothelium was demonstrated in each case by adding acetylcholine (10^{-4} mol/L) to the perfusion fluid and observing a relaxation in response to this endothelium-dependent vasodilator (Fig 2). Thrombin was then infused via the side branch at an initial rate of 1 U/mL (expressed as the final concentration after dilution in the perfusing stream). The response to thrombin was variable, but was consistently tachyphylactic; ie, when relaxation occurred, the tension would spontaneously
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The bath surrounding the vessel; it remains in contact with the vessel wall for the remainder of the experiment (the dose is reduced to 0.25 U/mL after the second wash). Note that whereas the response to thrombin is tachyphylactic, the response to acetylcholine is not. The heavy double bars represent relaxation of time. Hb (10⁻⁵ mol/L) is added to the perfusing solution, and acetylcholine (10⁻⁴ mol/L) is added to this mixture once the response to Hb had returned to its previous level after several minutes despite continued infusion of thrombin. The infusion rate of thrombin was then decreased to 0.25 U/mL and continued at that rate throughout the remainder of the experiment, except when platelets were studied with an aggregatory stimulus. Relaxations to acetylcholine, on the other hand, were not tachyphylactic, even after tachyphylaxis of the response to thrombin.

When RBCs, acetylcholine, or Hb were added to the perfusing solution, they were allowed to infuse long enough for the contractile or relaxant response to stabilize before platelets were infused.

Platelets (suspended in 4 mL of the perfusing solution) were infused for 2-minute periods. The effluent was collected into an equal volume of iced 0.1% glutaraldehyde to stop aggregation. The samples collected were processed for scintillation counting as described above under "Aggregometry" to determine fractional 5-hydroxytryptamine release.

Statistical analysis. All results are expressed as mean ± SE. Statistical testing was by two-way analysis of variance, using a random-block design to account for repeated measurements. Specific intergroup comparisons were made by Scheffe's test.⁷

RESULTS

Aggregometry studies. Thrombin (0.25 U/mL) was an effective agonist of platelet aggregation, as demonstrated by light transmission aggregometry or by the release of ¹⁴C-5-hydroxytryptamine measured simultaneously (Table 1). Neither aggregation nor 5-hydroxytryptamine release from platelets was inhibited by acetylcholine (10⁻⁶ mol/L) (n = 5). Hb (10⁻⁵ mol/L) likewise did not affect aggregation or release (n = 4), nor did the combination of Hb plus acetylcholine. RBCs added to the platelet suspension with hematocrits from 1% to 25% did not allow light transmission to be recorded, but platelet activation as judged by 5-hydroxytryptamine release was inhibited slightly (by 18.7% with a hematocrit of 10%, n = 7). This may have been due to interference with stirring, which would not occur in the perfused vessel. RBCs treated with echothiophate did not act differently from untreated RBCs in this regard.

Perfusion studies. When thrombin was infused via the cannulated branch vessel, platelet activation occurred as shown by the release of ¹⁴C-5-hydroxytryptamine (70% to 80%). However, when no thrombin was infused as a platelet agonist, the degree of platelet activation on transit through the perfused artery was minimal (¹⁴C-5-hydroxytryptamine release: 8.3% ± 2.4%, n = 5).

When acetylcholine was added to the perfusion solution, prompt relaxations of the vascular smooth muscle ensued. Additionally, platelet activation was significantly inhibited (¹⁴C-5-hydroxytryptamine release reduced by 17.5% ± 3.7% of the response to thrombin, n = 20 total experiments). This inhibitory effect was consistent and repeatable over the duration of an experiment for a given vessel (Fig 3). When Hb (10⁻⁵ mol/L) was added to the perfusion solution, a modest contraction of the vascular smooth muscle would usually occur. When acetylcholine was added in the presence of Hb, the relaxation evoked by acetylcholine was prevented (Fig 2). Hb caused no change in the degree of platelet activation by thrombin. However, Hb did abolish the inhibitory effect of acetylcholine on platelet activation (P < .05, n = 5) (Fig 4).

Echothiophate-treated RBCs, when added to the perfusion solution at a hematocrit of 10%, did not block the relaxation that occurred on the addition of acetylcholine, but did abolish the significant inhibitory effect exerted by acetylcholine on platelet aggregation (P < .05, n = 6) (Fig 4). In preliminary experiments, lower concentrations of RBCs (1% or 5% hematocrit) were insufficient to completely block acetylcholine's inhibitory effect (data not shown). The supernatant obtained after centrifugation of the perfusate containing RBCs was clear (in comparison with the marked red color imparted by 10⁻³ mol/L purified Hb), indicating that no major degree of hemolysis had occurred.

Table 1. Aggregometry Experiments

<table>
<thead>
<tr>
<th></th>
<th>Thrombin 0.25 U/mL (n = 5)</th>
<th>Unstimulated (n = 5)</th>
<th>Thrombin + Acetylcholine 10⁻⁶ mol/L (n = 5)</th>
<th>Thrombin + Hb 10⁻⁵ (n = 4)</th>
<th>Thrombin + Acetylcholine + Hb 10⁻⁵ (n = 4)</th>
<th>Thrombin (n = 8)</th>
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<tbody>
<tr>
<td>Aggregation</td>
<td></td>
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<tr>
<td></td>
<td>58.2 ± 3.7</td>
<td>0</td>
<td>56.4 ± 3.1</td>
<td>58.5 ± 3.7</td>
<td>60.5 ± 4.1</td>
<td>56.1 ± 3.0</td>
</tr>
<tr>
<td>¹⁴C-5HT release</td>
<td></td>
<td>5.9 ± 1.2</td>
<td>81.5 ± 4.8</td>
<td>84.2 ± 3.0</td>
<td>85.2 ± 2.7</td>
<td>81.3 ± 4.4</td>
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Responses are expressed as percentages.
DISCUSSION

The physiologic role of EDRF is not yet fully known. From observations that adenosine diphosphate and 5-hydroxytryptamine released from aggregating platelets can provoke endothelium-dependent relaxation in coronary arteries,\textsuperscript{8,12} it seems that one such role may be to promote vasodilatation at sites of platelet thrombus formation, and thereby protect arteries from occlusion.\textsuperscript{11} Inhibition of platelet aggregation by EDRF would further preserve blood flow. However, if the platelet-inhibitory action of EDRF were unregulated, it could cause a bleeding diathesis.

The current studies demonstrate that the endothelial cells of a perfused artery can inhibit the activation of platelets passing through the lumen. This inhibitory activity (triggered by acetylcholine) is almost certainly due to EDRF: the production of the other known endothelially derived platelet inhibitor, prostacyclin, is prevented by acetylsalicylic acid; and the inhibitory activity is blocked by Hb, a specific antagonist of EDRF activity.\textsuperscript{3} Experiments in the aggregometer confirm that acetylcholine does not directly inhibit platelet activation, nor does Hb augment it.

Several investigators have previously demonstrated that endothelium-derived relaxing factor can inhibit platelet aggregation\textsuperscript{3,4,14-20} and adhesion in vitro\textsuperscript{21-23} or in situ in a buffer-perfused lung.\textsuperscript{24} However, many of those studies have used cultured endothelial cells rather than intact vascular endothelium as the source of EDRF, and most have studied the platelets in the aggregometer. Two studies have demonstrated that systemic exposure to a large dose of carbamylcholine to release EDRF in vivo can inhibit platelet function (assessed by pulmonary platelet trapping\textsuperscript{25} or in vitro aggregometry\textsuperscript{26}); however, in those experiments one presumes that the entire body’s endothelium has been activated. Thus, until now it has remained moot whether a conduit artery’s endothelium in situ could release enough EDRF locally to inhibit platelets flowing through that artery. The present experiments confirm that it can.

Free Hb can block the platelet-inhibiting action of locally released EDRF. Whole blood contains about 200 to 250 times as much Hb as was required, in cell-free form, to abolish the platelet inhibition. However, it has not been clear whether the Hb contained within erythrocytes is available to bind EDRF. One study\textsuperscript{27} using a cascade bioassay system found that a concentration of rat RBCs “equivalent to 10^{-6} mol/L hemoglobin” could abolish endothelium-dependent relaxation when added downstream from the donor but upstream from the assay tissue. This number of RBCs would amount to a hematocrit of only about 0.02%. On the other hand, two studies\textsuperscript{25,26} have been able to demonstrate inhibition, presumed to be due to EDRF, of platelet aggregation in whole blood using electrical impedance-aggregometry.

The present experiments show that RBCs can block platelet inhibition by EDRF. Because a 10% hematocrit is sufficient to do so, it would appear that the endothelium of a large artery perfused in vivo by whole blood with a normal hematocrit would not be able to release enough EDRF to abolish platelet thrombus formation intraluminally. Even so, EDRF may still be able to inhibit platelet adhesion to the endothelial surface (and hence prevent initiation of thrombus formation): as erythrocytes tend to stream to the center of the lumen, platelets in the immediate vicinity of the endothelial cell may be exposed to inhibitory concentrations of EDRF. Furthermore, synergistic interaction between the antiaggregatory effects of EDRF and prostacyclin\textsuperscript{15,18,19} may amplify the effect of EDRF.

In contrast to their effect on platelet inhibition, RBCs do not block the relaxation of arterial smooth muscle evoked by EDRF release in response to acetylcholine. This is in keeping with in vivo observations of endothelium-dependent vasodilatation in blood-perfused arteries.\textsuperscript{28} In contrast, the fact that free Hb can block smooth muscle relaxation may indicate that Hb can gain access to the subendothelial space and bind EDRF there; intact RBCs, needless to say, cannot do so.

The lack of inhibition of relaxation by intact RBCs demonstrates two other points. First, the blockade of acetylcholine’s inhibitory effect is not merely an artifact due to breakdown of acetylcholine by the RBCs\textsuperscript{29,30}; this confirms that treatment with echothiophate has effectively blocked.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Results of a perfusion experiment, in which platelet activation in transit through the vessel is reflected by the percentage of $^{14}$C label released into the supernatant of the perfusate collected downstream. In all but the last column ("Unstimulated"), thrombin is infused via a proximal side branch to activate the platelets. "ACh" signifies the addition of acetylcholine ($10^{-6}$ mol/L) to the perfusion solution (light columns) during thrombin infusion.}
\end{figure}
Platelet activation during perfusion. In all columns, thrombin is infused via a proximal side branch. “ACh” signifies the addition of acetylcholine to the perfusion solution (light columns). (A) “Hemoglobin” represents the addition of Hb (10^-5 mol/L) to the perfusion solution (overlying bar). The inhibitory effect of acetylcholine is blocked in the presence of Hb (P < .05), but recovers after Hb is withdrawn from the perfusion solution. (B) “RBC’s” represents the addition of erythrocytes (10% hematocrit) to the perfusion solution (overlying bar). Platelet activation is significantly inhibited by acetylcholine (P < .05), and this effect is abolished by the presence of RBCs.

Fig 4.

erythrocyte acetylcholinesterase. Second, the effect of RBCs is not due simply to hemolysis and release of free Hb (although we cannot rule out the possibility that a slight degree of hemolysis may have occurred, which may have contributed somewhat to antagonizing the EDRF effect).

It is of interest that a hematocrit on the order of 10% appears necessary to block completely the platelet inhibition by EDRF. This is 40 to 50 times the concentration of free Hb needed to do so. This would suggest that the Hb contained within RBCs is in some way unable to freely interact with EDRF, even though it is generally supposed that nitric oxide (the putative chemical identity of EDRF\(^1\)) should be freely permeable through cell membranes. Streaming of RBCs to the center and platelets to the periphery of the lumen may explain this apparent discrepancy.

An intriguing speculation arises from the current result that a relatively large concentration of RBCs is needed to block the platelet-inhibiting action of EDRF. In anemia, platelets may be exposed to an increased tonic inhibitory effect of EDRF, and the bleeding time thereby prolonged. Such an inverse correlation between hematocrit and bleeding time has long been recognized clinically,\(^{32,34}\) although it has never been fully explained.

In summary, EDRF released from a conduit artery’s endothelium can inhibit the activation of platelets in transit through the vessel. This effect, at least in large-caliber vessels, is blocked by the presence of RBCs.

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REFERENCES


Inhibition of intravascular platelet aggregation by endothelium-derived relaxing factor: reversal by red blood cells

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